Novel Pathways of Regulation of the Transcription Factor Spx in *Bacillus Subtilis*

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ABSTRACT

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Chair of the Supervisory Committee:

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Spx is a transcription factor present in low G+C Gram-positive bacteria, including *Bacillus subtilis* and several human pathogens. By direct binding to the αCTD domain of the RNA polymerase, Spx modulates the expression of a large set of genes in *B. subtilis*. The Spx regulon is active in growing cells, and its expression is further induced in response to some stresses. Spx activation has been typically studied using diamide to generate disulfide stress. By using molecular biology, genetic, and biochemistry techniques, I show that the Spx regulon is also induced in response to cell wall stress, and that Spx is critical for survival of *B. subtilis* upon treatment with cell wall antibiotics. I further show the molecular mechanisms that lead to activation of Spx. Unlike disulfide stress, induction of the Spx regulon in response to cell wall stress requires transcriptional induction of the *spx* gene. This induction is mediated by the alternative extracytoplasmic sigma factor σ^M, and occurs at a promoter upstream of the *yjbC-spx* operon (i.e. P_{M1}). Interestingly, activation of the Spx regulon in response to cell wall stress also requires stabilization; however, unlike disulfide stress, this process is mediated by a small protein called YirB. YirB is an anti-adaptor protein that binds with high affinity to YjbH, the adaptor protein required for ClpXP-mediated proteolysis, and therefore reduces the rate of Spx degradation. Transcriptional induction and post-translational stabilization are thus required for activation of the Spx

regulon in response to antibiotics that inhibit peptidoglycan biosynthesis. Then, I show that *yirB* is also induced in response to cell wall stress, and that its activation requires the coordinated action of the YuxN repressor and the CssRS two-component system. Finally, I show that Spx is not only degraded through ClpXP, but also by ClpCP. The adaptor that mediates this degradation in unstressed cells appears to be MecA, and the evidence comes from the Spx-dependent synthetic lethality of ClpX and MecA. The McsB arginine kinase, which also acts as a ClpCP adaptor, as well as the YwlE arginine phosphatase are also shown to play an important role in Spx regulation. Overall, this work expands the regulatory mechanisms that control the activity of a pleiotropic transcription factor in *B. subtilis*.

DEDICATION

This work is dedicated to my parents Lesma Tapias and Fernando Rojas, my brother Rodian Rojas, my girlfriend Carolina Mazo, my grandmothers Cecilia Ibarra and Beatriz Vargas, my cousin Juan Agudelo, and all my family and friends, for their love and support throughout my doctoral studies and scientific career.

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BIOGRAPHICAL SKETCH

Born and raised in Bogotá, Colombia, Daniel Rojas is a Ph.D. candidate at the Department of Microbiology at Cornell University. Daniel's research focuses on the regulatory mechanisms that control the activation of the transcription factor Spx in *Bacillus subtilis*. In particular, he found that Spx is induced in response to and is important for the adaptation of *B. subtilis* cells to cell wall antibiotics. Daniel uncovered the genetic pathways that lead to the activation of the Spx regulon in response to inhibitors of peptidoglycan synthesis.

Prior to attending Cornell, he worked at the Center of Biotechnology and Bioindustry at the Colombian Corporation for Agricultural Research - Agrosavia. His research at Agrosavia focused on understanding how beneficial microorganisms could be used to ameliorate the negative impact of different abiotic stresses on plant growth, in particular, salinity and heavy metals.

Daniel earned a bachelor's degree in Microbiology at Pontifical Xavierian University in Bogotá, Colombia. During his undergraduate, Daniel used sequential statistical designs for the formulation of culture media for massive production of biofertilizers. He also earned a bachelor's degree in Mathematics at the same university. He studied the mathematical basis of the multivariate statistical technique of principal component analysis. Then, he pursued a master's degree in Microbiology at The University of Andes in Bogotá, Colombia. During his master, he investigated the use of bacteria for phytoremediation of heavy metals in contaminated soils.

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SUMMARY

Spx is a redox-responsive transcription factor present in low GC Gram-positive bacteria, including *Bacillus subtilis*. By direct binding to the αCTD domain of the RNA polymerase, Spx modulates the expression of a large set of genes in *B. subtilis*, and whereas most genes in the Spx regulon are positively regulated, some are repressed. Spx interacts with the RNA polymerase holoenzyme harboring the housekeeping σ^A sigma factor. The Spx regulon is active in growing cells, and its expression is further induced in response to some stresses. I present the regulation and function of Spx in *B. subtilis* and other Firmicutes in Chapter I.

Although most of our understanding of *B. subtilis* Spx comes from studies using diamide as a tool to generate disulfide stress, a condition that results in accumulation and oxidation of Spx, recent investigations have shown that additional stress conditions (e.g. heat and ethanol) also result in induction of the Spx regulon. In this study, I show that cell wall stress also leads to activation of the Spx regulon, and that cells lacking Spx display increased sensitivity towards antibiotics that inhibit the synthesis of peptidoglycan (Chapter II). Interestingly, the mechanisms that result in activation of the Spx regulon in response to cell wall stress differ from those mechanisms observed in response to disulfide stress.

In unstressed cells, the Spx protein is highly unstable due to active ClpXP-mediated proteolysis, and therefore the protein concentration is low. In response to disulfide stress, however, the protein accumulates and oxidizes, leading to a dramatic increase in the expression of Spx-controlled genes. The induction of the Spx regulon under these conditions solely relies on post-transcriptional events in spite of the fact that the *spx* gene displays a complex transcriptional context. In this study, we show that, unlike disulfide stress, induction of the Spx regulon in response to cell wall stress requires induction of the *spx* gene. This induction is mediated by the alternative sigma factor σ^M (Chapter II).

The activation of the Spx regulon in response to disulfide stress requires proteolytic stabilization, which takes place through inactivation of the ClpX unfoldase and aggregation of the YjbH adaptor protein. This molecular strategy permits Spx accumulation in absence of changes in *spx* expression. Interestingly, activation of the Spx regulon in response to cell wall stress also requires stabilization; however, unlike disulfide stress, this process is mediated by a small protein called YirB. YirB is an anti-adaptor protein that binds YjbH, the adaptor protein required for ClpXP-mediated proteolysis, and therefore reduces Spx proteolysis. Transcriptional induction and post-translational stabilization are required for activation of the Spx regulon (Chapter III).

yirB itself is induced by the CssRS two-component system in response to cell wall stress. This process takes place rapidly after stress and is critical for the early accumulation of Spx in response to vancomycin. In this system, unexpectedly, CssRS does not act as an inducer for *yirB* transcription; instead, it acts as an anti-repressor protein. Under unstressed conditions, the expression of *yirB* is repressed by YuxN, a TetR-like repressor located downstream *yirB*, which prevents the activation of *yirB* and *yuxN* itself. Under vancomycin treatment, CssRS is induced and CssR~P prevents binding of YuxN to DNA. This occurs since both YuxN and CssR~P bind overlapping DNA boxes. Expression of *yirB* leads to Spx stabilization (Chapter III).

Activation of the Spx regulon in response to cell wall stress therefore relies on transcriptional and post-translational events. Over the course of these experiments, however, we noticed that some experimental observations did not fit the known models for Spx regulation, suggesting that additional pathways were involved. By using transposon mutagenesis, we observed that deletion of CtsR, the master regulator of proteolysis, resulted in reduced induction of Spx-controlled genes. Analysis of this mutant revealed that *in vivo* Spx proteolysis can occur in a ClpXP-independent fashion. Genetic experiments revealed that ClpC, another member of the HSP100 family of proteases, also can target Spx for degradation through the ClpP protease. Spx degradation via ClpCP, however, occurs at a lower rate. We also provide evidence for a connection between Spx regulation and arginine

phosphorylation. Finally, we show that the autoregulated *ctsR* operon is also under Spx control, which reveals a negative feedback loop for Spx activity (Chapter IV).

In *B. subtilis*, the Spx regulon is activated in response to multiple environmental conditions. The regulatory pathways involved in its activation display remarkable complexity, and require the integration of multiple signals. And its activation is critical for survival in response to various stress conditions. In this work, we further expand the biological role and regulatory mechanisms associated to Spx control in *B. subtilis*.

CHAPTER I: Regulation of the transcription factor Spx in *Bacillus subtilis* and related species

1.1 Summary

Spx is a protein belonging to the ArsC family of transcriptional regulators. Spx modulates gene expression by direct binding to the αCTD domain of the RNA polymerase harboring the σ^A sigma factor. In *B. subtilis*, Spx drives the expression of a vast regulon in response to proteotoxic conditions, such as heat and disulfide stress, as well as cell wall stress. The molecular mechanisms that result in its activation are, however, different in response to those conditions. For instance, activation of the Spx regulon in response to disulfide stress relies on reduced Spx proteolysis and oxidization of the Spx redox-sensing switch, whereas activation in response to cell wall antibiotics necessitates both transcriptional and post-translational control. A detailed revision of the current knowledge regarding the molecular mechanisms that lead to *B. subtilis* Spx activation in response to several stress conditions is the main topic of this review.

The family of Spx proteins, however, is not restricted to *B. subtilis.* Spx homologs are also found in other genera within the Firmicutes phylum. The number of homologs dramatically varies among species. For instance, in addition to Spx, *B. subtilis* contains one additional Spx paralog (i.e. MgsR), which binds and modulates the activity of the RNA polymerase containing the σ^B sigma factor. In other species, the number of Spx homologs is smaller. For instance, *Listeria monocytogenes* and *Staphylococcus aureus* possess only one Spx homolog. By contrast, species such as *Lactococcus lactis* and *Lactococcus garvieae* contain six and eleven paralogs, respectively. The typical features that characterize Spx also differs among paralogs, and thus some canonical *B. subtilis* Spx features are not present in other Spx proteins. The study of the different Spx homologs throughout representative Firmicutes is also the focus of this review.

This review also presents a description of the current knowledge regarding the regulation and function of Spx homologs in other Firmicutes. The goal is to provide an overview of how Spx proteins are regulated in other species, the environmental conditions in which those homologs are activated, and their biological role. The current evidence suggests, for instance, that the function of Spx proteins is broader than previously anticipated, and that its role is not restricted to oxidative stress. For example, in *L. lactis*, the SpxA6 protein modulates the expression of genes involved in peptidoglycan modification, and its overexpression protects the cells against lysozyme treatment.

Finally, we provide some perspectives, and describe some areas in which further research is required. Given the importance of Spx in some pathogenic and economically important Firmicutes, a comprehensive study of the regulation and function of the members of this family of proteins is critical for future biological and biotechnological investigations.

1.2 Introduction

Spx is a transcription factor present in *Bacillus subtilis* (Nakano et al., 2003a), and other Gram-positive bacteria within the Firmicutes phylum (Frees et al., 2001; Kajfasz et al., 2010; Pamp et al., 2006; Whiteley et al., 2017). Spx is active in growing cells, but strongly induced in response to different stress conditions such as disulfide, heat, and cell wall stress (Nakano et al., 2003a; Rojas-Tapias and Helmann, 2018a; 2018b; Runde et al., 2014). Spx regulates the expression of a large number of genes, with most of them subject to transcriptional activation, with others repressed (Nakano et al., 2003a; Rochat et al., 2012). The *spx* gene was discovered as the locus for mutations that restored competence in cells lacking ClpX or ClpP; Spx stands for suppressor of ClpP and ClpX (Nakano et al., 2001). The Spx protein was identified as a transcription factor belonging to the ArsC family and found to be important for the disulfide stress response in *B. subtilis* (Nakano et al., 2003a). Spx is the key regulator of the thioredoxin system in *B. subtilis*, and a repressor of the *srfAA* operon. Cells lacking

Spx are more sensitive to treatment with diamide, an electrophilic compound that leads to formation of intracellular disulfide bonds (Nakano et al., 2003a). Spx was also reported to act as an anti-alpha factor that blocks transcriptional activation by binding to the C terminus of the RNA polymerase's alpha domain. By binding to this domain, Spx blocks the activity of other transcription regulators required to induce gene expression, including genes required for competence development (Nakano et al., 2003b).

The *spx* gene has been identified in other low-GC Gram-positive bacteria in the Firmicutes phylum, including some human pathogens such as *Staphylococcus aureus* (Pamp *et al.*, 2006), *Enterococcus faecalis* (Kajfasz *et al.*, 2012), and *Streptococcus mutans* (Kajfasz et al., 2010; 2015). In those organisms, the general function of Spx seems to be similar to that in *Bacillus subtilis* and involves roles related to oxidative stress. In some organisms, including *B. subtilis*, Spx also appears to be important for the cell wall stress response (Jousselin et al., 2013; Renzoni et al., 2011; Rojas-Tapias and Helmann, 2018a; Veiga et al., 2007). Interestingly, different numbers of Spx paralog proteins are present in different species, which might suggest a specialized role for different Spx proteins. For instance, *S. aureus*, *B. subtilis*, and *Lactococcus lactis* contain one, two, and six Spx-like proteins, respectively.

In recent studies in *B. subtilis*, it has been demonstrated that Spx and Spx-controlled genes are induced by a wider variety of stress conditions than initially appreciated, including conditions that result in protein misfolding and aggregation such as ethanol or heat stress (Engman and Wachenfeldt, 2015; Runde et al., 2014). In addition, the regulon is induced, for example, under conditions in which the integrity of the cell wall is threatened, such as exposure to cell wall-active antibiotics such as fosfomycin or vancomycin which prevent peptidoglycan assembly (Rojas-Tapias and Helmann, 2018a; 2018b). Spx-controlled genes are similarly induced under active growth, and they are predicted to also be induced under secretion stress conditions.

The major function of the Spx stress response is to prevent and alleviate protein aggregation and misfolding (Rochat *et al.*, 2012). Accordingly, the Spx regulon confers resistance against disulfide and heat stress. Consistent with its role in preventing protein aggregation, Spx controls the activity of protein chaperones and proteases, genes involved in synthesis of low-molecular-weight thiols, and other proteins predicted to be important in the oxidative stress response (Gaballa et al., 2013; Nakano et al., 2003a; Rochat et al., 2012). The Spx regulon also confers resistance against cell wall stress, but the associated mechanisms remain unclear (Rojas-Tapias and Helmann, 2018a). Although the role of many genes in the Spx regulon still remains unknown, in the past years much has been learned regarding the regulation of Spx in response to different stresses. In this review, we will consider the mechanisms that result in activation of the Spx regulon in *B. subtilis* in detail, and consider what is known regarding Spx and its role in other species. Additionally, we will revisit the importance of Spx in light of new insights into the composition of its regulon and phenotypes associated with *spx* mutants.

1.3 *B. subtilis* Spx is a global regulator with pleiotropic effects

In *B. subtilis,* Spx is a global regulator that drives the expression of 144 transcriptional units in response to disulfide stress (Rochat *et al.*, 2012). Spx plays an important adaptive role in response to stress conditions, as cells lacking it display increased sensitive to heat shock (Runde *et al.*, 2014), diamide stress (Nakano et al., 2003a), and some cell wall-active antibiotics (Rojas-Tapias and Helmann, 2018a). Excessive Spx also has a negative effect on several physiological processes including growth, competence, and sporulation (Larsson et al., 2007; Nakano et al., 2001). Cells must therefore tightly control the concentration of Spx to efficiently activate stress responses without causing detrimental effects on bacterial growth and development.

The Spx-controlled genes involved in the adaptation to different stresses are fairly well understood in the case of disulfide stress, but less clear for cell wall antibiotics. For instance, the

increased sensitivity of *spx* null mutants to diamide is explained by the inability of *Δspx* cells to induce the thioredoxin system. This evidence emerged from complementation experiments wherein the resistance of a *Δspx* null mutant to diamide was restored with the expression of a synthetic DNA construct containing the thioredoxin (i.e. *trxA*) and thioredoxin reductase (i.e. *trxB*). The genes involved in resistance to cell wall stress are less known; however, complementation with the *trxA-trxB* synthetic operon increases 10 times the survival of an *Δspx* mutant challenged with cycloserine and ampicillin, but not fosfomycin (unpublished data). This observation seems to suggest that under cell wall stress conditions some proteins involved in peptidoglycan synthesis might be inactivated by oxidation of specific cysteine residues.

The effect of Spx overexpression on *B. subtilis* cells seems to be more complex, as it likely involves overexpression and repression of Spx-controlled genes, as observed in a strain harboring the spx^{DD} allele (Nakano et al., 2003a), as well as interference with activator-stimulated transcription (Nakano et al., 2003b). Whether overexpression and/or repression of the genes in the Spx regulon results in any negative impact on bacterial growth is yet unclear; by contrast, the effect of elevated Spx levels on the induction of activator-regulated genes is well understood (Nakano et al., 2003b). By interacting with the C-terminal domain of the RNA polymerase α-subunit, Spx blocks binding of other transcription regulators and therefore affects transcription. This effect is well known in the case of the *srf* promoter and ComA, in which Spx prevents binding of the response regulator ComA to the *srf* promoter and thus prevents its transcription. Since the *srf* promoter regulates the expression of *comS*, an anti-adaptor protein with an essential function in competence development, cells overexpressing Spx are not capable of taking up external DNA (Nakano et al., 2003b).

The activation of the Spx regulon is multifactorial as it relies on changes in transcription, proteolysis, and/or post-translational modifications. At the transcriptional level, *spx* induction is regulated by the coordinated action of several promoters controlled by different sigma factors (Antelmann et al., 2000; Jervis et al., 2007; Leelakriangsak and Zuber, 2007; Rochat et al., 2012;

Rojas-Tapias and Helmann, 2018a), as well as two protein repressors (Leelakriangsak *et al.*, 2007). The transcription of *spx* is known to be stimulated by phosphate starvation (Antelmann *et al.*, 2000), oxidative stress (Kawai *et al.*, 2015), and cell wall stress (Rojas-Tapias and Helmann, 2018a), although *spx* is also potentially induced in response to heat shock, ethanol and salt stress, as well as in biofilms (Nicolas *et al.*, 2012). Once Spx is synthesized, its concentration can be controlled by regulated degradation, which involves the action of proteases, adaptors, and one antiadaptor protein (Garg et al., 2009; Rojas-Tapias and Helmann, 2018a; Zhang and Zuber, 2007). In cells growing on LB media, for instance, the concentration of the Spx protein is low, as Spx is degraded with a half-life of ~2 min. Under stress conditions, however, Spx proteolysis is considerably reduced or even completely inhibited, which allows for Spx accumulation and induction of the regulon (Rojas-Tapias and Helmann, 2018b; Zhang and Zuber, 2007). Finally, at the post-translational level, the protein can form a disulfide bond at its redox-sensing switch, which greatly increases protein activity (Nakano et al., 2005; Rochat et al., 2012; Rojas-Tapias and Helmann, 2018a).

1.4 Regulation of *spx* transcription

The *spx* gene is highly transcribed in unstressed cells growing in rich medium, and its expression level is only slightly affected throughout growth (Nicolas *et al.*, 2012). *spx* is the second gene in a bicistronic operon also composed by *yjbC*, a gene that encodes a putative acetyltransferase with a still unknown function. *spx* transcription is modulated by the activity of several promoters located upstream *yjbC* or within the *yjbC-spx* intergenic region. These promoters are recognized by different holoforms of the RNA polymerase and hence induced under distinct conditions. In addition to these promoters, two protein repressors modulate the transcription of *spx* (Fig 1.1). Transcriptomic studies using a ChIP-tiling-microarray showed that *spx* is not subjected to autoregulation, and therefore deletion of the *spx* gene has no effect on its own transcription (Rochat *et al.*, 2012). The same analysis

also predicted the existence of two promoters in the non-sense DNA strand: one is located in the intergenic region of *yjbC-spx* and another within the *spx* coding region; these promoters might presumably lead to the synthesis of antisense RNAs to modulate *spx* mRNA levels. Since the regulatory events that result in induction of the Spx regulon in response to disulfide stress occur at the post-translational levels, the importance of *spx* transcriptional control in induction of the regulon has been comparatively little studied. Recently, however, the importance of one of those promoters in induction of the Spx regulon in response to cell wall stress was defined (Rojas-Tapias and Helmann, 2018a).

1.4.1 Promoters contributing to *spx* transcription

The expression of the *yjbC-spx* operon is controlled by two different promoters: P_{M1} and P_B (Fig 1.1). The induction of P_{M1} was first detected using an inducible allele of *sigM* under xylose control and a library of *lacZ* transcriptional fusions (Jervis et al., 2007). Interestingly, the *yjbC-spx* promoter showed the highest beta-galactosidase levels among all the promoters tested. The promoter sequence of P_{M1} was mapped using 5'-RACE-PCR and shown to display all the typical features of ECF sigma factor-controlled promoters. Previous studies had identified P_{M1} as a σ^W-regulated gene (Huang *et al.*, 1999). The P_{M1} promoter is induced in response to cell wall antibiotics in a σ^M-dependent fashion and is required for the induction of the Spx regulon in response to cell wall stress (Rojas-Tapias and Helmann, 2018a). Cells harboring an inactivated P_{M1}* promoter therefore display reduced Spx levels in response to cell wall antibiotics. σ^X is also able to induce P_{M1} in response to cell wall-antibiotics, but only in absence of σ^M; the conditions in which σ^X by itself induces *spx* are still unknown. The P_B promoter, on the other hand, is induced in response to phosphate limitation (Antelmann *et al.*, 2000), and presumably -ethanol and salt stress (Nicolas *et al.*, 2012). However, whether its induction results in activation of Spx-controlled genes remains unknown. A third promoter upstream of P_B was

identified in previous studies, but its sequence does not resemble any known promoter motif in *B. subtilis* (Antelmann *et al.*, 2000).

The *spx* gene itself is also transcriptionally induced from two promoters located in the intergenic region of the *yjbC-spx* operon. The P_{M2} promoter is controlled by σ^M, but its induction has only been observed in an engineered strain with conditional expression of σ^M (Jervis *et al.*, 2007). Importantly, cell wall stress does not lead to induction of this promoter, and therefore the physiological relevance of this promoter has not been defined (Rojas-Tapias and Helmann, 2018a). Additionally, a σ^A-controlled promoter (i.e. P_A) is located downstream of P_{M2} (Leelakriangsak and Zuber, 2007). This promoter is constitutively active and is sufficient to complement an *Δspx* null mutant with respect to diamide resistance. The P_A promoter is subjected to regulation by two protein repressors (see below), which modulate its activity in response to stress.

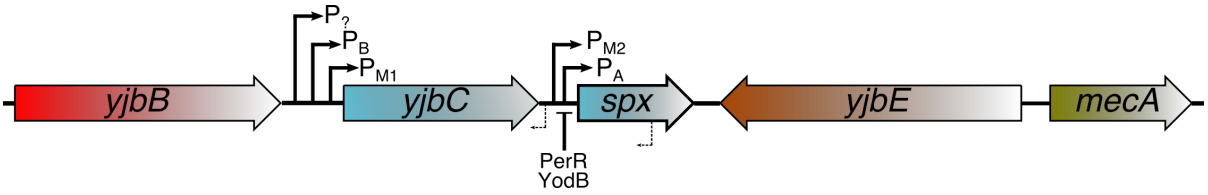

FIG 1.1 REGULATION OF SPX TRANSCRIPTION AND GENETIC CONTEXT IN BACILLUS SUBTILIS.

A total of five promoters and two protein repressors have been predicted to drive the expression of *B. subtilis spx*.

1.4.2 Protein regulators controlling *spx* expression

The activity of the P_A promoter is regulated by two trans-acting regulators that repress *spx* expression: PerR and YodB (Leelakriangsak *et al.*, 2007) (Fig 1.1). Initial observations that linked the expression of *spx* to PerR emerged from the study of the *srf* operon in *B. subtilis.* Hayashi *et al.* noticed that inactivation of *perR* not only caused downregulation of the *srf* operon, but also an ~10-fold increase in *spx* expression as compared to wild-type (Hayashi *et al.*, 2005). Further studies revealed

that not only PerR, but also YodB, can repress *spx* at P_A; a promoter that is active during both exponential and stationary phases (Leelakriangsak *et al.*, 2007). The effect of PerR and YodB on *spx* transcription is additive, as a mutant lacking both regulators displayed increase *spx* expression compared with cells lacking either *perR* or *yodB* (Leelakriangsak *et al.*, 2007). Stress conditions that result in derepression of either regulator are therefore expected to affect *spx* expression and potentially activate the Spx regulon.

PerR is a repressor in the Fur family that responds to hydrogen peroxide (Herbig and Helmann, 2001). PerR senses hydrogen peroxide by Fe^{+2}-catalyzed oxidation of the His37 or His91 residues located at the metal-binding site that coordinates the Fe^{+2} cofactor. The oxidation of either His residue results in reduced DNA binding and thus activation of the regulon (Lee and Helmann, 2006). Members of the PerR regulon include the vegetative catalase (*katA*), alkyl hydroperoxidase (*ahpC*), the iron efflux transporter (*pfeT*), *spx*, and *perR* itself (Faulkner and Helmann, 2011; Fuangthong et al., 2002; Leelakriangsak et al., 2007). The role of the PerR regulon is thus to allow the cells to cope with the oxidative stress generated by hydrogen peroxide. By modulating *spx* levels, PerR can also activate other systems required to cope with this oxidative stress conditions, including the thioredoxin system and the Clp proteases. Induction of the P_A promoter in a PerR-dependent fashion has been observed in protoplasts of *B. subtilis* cells undergoing oxidative stress (Kawai *et al.*, 2015).

YodB is a protein regulator that belongs to the MarR/DUF24 family of repressors and controls the expression of *azoR1*, *catDE*, *yodC,* and *spx* (Chi et al., 2010; Leelakriangsak et al., 2007; 2008). Diamide and quinones are able to activate YodB , an event that occurs by modification of the Cys6 and/or Cys101 residues (Leelakriangsak *et al.*, 2008). *B. subtilis* YodB uses two distinct pathways to regulate transcription in response to diamide and quinones: diamide affects the structural arrangement of the YodB dimer through formation of disulfide bonds, while quinones allow the dimer to be release from DNA without major conformational changes (Lee *et al.*, 2007). This differential activation mechanisms allows the activation of YodB-controlled genes by different chemicals at different

concentration; activation of *azoR1*, for instance, is maximal at 0.01 mM methyl-p-benzoquinone, while 10 mM diamide is required to cause the same effect (Lee *et al.*, 2007). This result, in fact, coincides with previous observations that show that the P_A promoter of *spx* fails to be induced by 0.5 mM diamide (Rochat *et al.*, 2012; Rojas-Tapias and Helmann, 2018a). And it also implies that quinones should be capable of activating the Spx regulon through derepression of P_A.

1.5 Regulation of Spx proteolysis

The *spx* gene is highly transcribed during exponential and stationary phase, but further upregulated in response to specific stress conditions (Antelmann et al., 2000; Eiamphungporn and Helmann, 2008; Kawai et al., 2015; Rochat et al., 2012; Rojas-Tapias and Helmann, 2018a). In most conditions, however, the concentration of Spx is low due to active proteolysis (Nakano et al., 2002). It is therefore expected that either or both a decrease in Spx proteolysis, as well as an increase in Spx synthesis (discussed above), can result in Spx accumulation. This is indeed the case, as reduced Spx degradation permits an increase in Spx accumulation in response to disulfide and cell wall stress, respectively. In *B. subtilis*, proteolysis of Spx involves a complex regulatory machinery that encompasses different proteases, adaptors, and anti-adaptor proteins (Fig 1.2).

1.5.1 Spx degradation by the ClpXP protease

In *B. subtilis,* Spx degradation primarily occurs through the ClpXP protease (Nakano et al., 2002) (Fig 1.2). ClpXP is barrel-shaped processive protease consisting of the ClpX unfoldase and ClpP protease. ClpX subunits are assembled in a hexameric ring, and its function is to discriminate the protein substrate and unfold it prior to transit into the proteolytic chamber which is composed by heptameric rings of ClpP. The activity of ClpXP is reduced by conditions that result in formation of disulfide bonds, and therefore ClpXP-mediated proteolysis of Spx is restricted in cells treated with

diamide. This effect is not limited to Spx, as diamide treatment also leads to *in vivo* and *in vitro* accumulation of SsrA-tagged proteins (Zhang and Zuber, 2007). The activity of ClpX *in vitro* is dramatically reduced in the presence of diamide or hydrogen peroxide, but unaffected in response to DTT, which shows that ClpX itself is affected by oxidative stress. In fact, Cys-to-Ser substitutions at positions 16 and 35, which comprise the Cys4 zinc-binding domain (ZBD), render ClpX insensitive to diamide treatment, suggesting that the formation of disulfide bonds in this domain affects ClpX activity. The inhibition of ClpXP activity in response to oxidative stress is thus the result, at least in part, of structural changes in its N-terminal ZBD domain. As expected, cells lacking ClpX or ClpP display increased levels of Spx, elevated transcription of Spx controlled genes, and repression of other transcriptional units. Interestingly, although *in vitro* Spx degradation by ClpXP could be reconstituted, the process occurred slowly. This observation suggested that additional factors might be critical for efficient Spx degradation.

1.5.2 ClpXP-dependent Spx proteolysis *in vivo* requires the YjbH adaptor protein

Although Spx can be proteolyzed *in vitro* by ClpXP this process is greatly increased in the presence of YjbH (Fig 1.2). Cells lacking YjbH display increased Spx levels and similar pleiotropic phenotypes (e.g. small colonies, reduced sporulation, and defective genetic competence) as those observed in cells lacking *clpX* or *clpP* (Larsson *et al.*, 2007). The *yjbH* gene was originally discovered as part of a screen to identify genes that provide increased survival of *B. subtilis* cells exposed to nitrosative stress (Rogstam *et al.*, 2007). Deletion of the *yjbI-yjbH* operon resulted in a significant decrease in survival compared to WT, and this defect could only be restored by ectopic expression of either *yjbH* or *yjbI-yjbH* (Rogstam *et al.*, 2007). Analysis of *ΔyjbH* suppressor mutants that reverted to the wild-type phenotype revealed mutations within the *spx* gene and its promoter region, suggesting that loss of Spx function or reduced synthesis accounted for the phenotype (Larsson *et al.*, 2007).

Indeed, transcriptomic analysis of *ΔyjbIH* mutants showed a striking similarity with microarray data from a strain overexpressing Spx^{DD} (a resistant-to-proteolysis Spx protein) (Nakano et al., 2003a), further suggesting a connection between YjbIH and Spx (Larsson *et al.*, 2007). This same analysis also showed that YjbH affects Spx at the post-translational levels, as *spx mRNA* levels are unaffected by deletion of *yjbIH*, while Spx levels are dramatically increased (Larsson *et al.*, 2007).

In vivo and in vitro analysis using yeast two-hybrid analysis and protein-protein crosslinking with purified Spx and YjbH, respectively, showed that these two proteins physically interact (Garg *et al.*, 2009). Interestingly, a YjbH mutant protein lacking its first 24 amino acids (i.e. $YjbH^{\Delta 1\text{-}24}$) was unable to bind Spx, suggesting a role for the N terminus in Spx binding; this N-terminal region consists of a histidine-rich region capable of coordinating one Zn atom that can be released under oxidizing conditions (Garg *et al.*, 2009). Indeed, Zuber and coworkers showed that *in vitro* Spx proteolysis by ClpXP, but not ClpCP, was enhanced by addition of YjbH, but not $YjbH^{\Delta 1\text{-}24}$. Furthermore, they showed that the function of YjbH was to specifically deliver Spx to ClpXP, and not increase ClpXP processivity, by showing that a SsrA-His6-GFP was degraded at similar rates in presence or absence of YjbH (Garg *et al.*, 2009).

YjbH functions as an the adaptor protein for ClpXP-mediated Spx proteolysis (Garg et al., 2009; Larsson et al., 2007): deletion of *yjbH* led to Spx accumulation without changes in *spx* transcription (Larsson *et al.*, 2007), Spx and YjbH physically interact *in vivo* (Garg *et al.*, 2009), and YjbH specifically increases Spx proteolysis *in vitro* through ClpXP but not ClpCP. Like other adaptor proteins (Battesti and Gottesman, 2013), YjbH plays a critical regulatory role as it permits rapid changes in Spx concentration in response to specific stress conditions (Engman and Wachenfeldt, 2015). YjbH is prone to aggregation and therefore conditions that result in protein aggregation and/or misfolding, such as disulfide or heat stress, result in its inactivation (Engman and Wachenfeldt, 2015). YjbH inactivation leads to Spx aggregation. Importantly, *B. subtilis* cells harboring a functional *yjbH* allele from the thermophilic Firmicutes *Geobacillus kaustophilus,* which is less prone to aggregation,

accumulate significantly less Spx in response to heat, ethanol, and disulfide stress. This piece of evidence clearly showed that YjbH aggregation is a key element that leads to Spx accumulation in response to these stresses (Engman and Wachenfeldt, 2015).

The recognition elements in Spx that allow YjbH binding and ClpXP degradation are located in the C-terminus region of Spx (Chan *et al.*, 2012). Point mutations in the amino acids in the conserved helix α6 abolish YjbH binding, and thereby protect against ClpXP proteolysis, as shown using an AbrB chimeric protein harboring the last 28 residues of Spx (Chan *et al.*, 2014). This Spx C-terminal region includes the ClpX recognition site, which is exposed in the chimeric protein but hindered in Spx due presumably to interactions with the core regions of Spx. Since YjbH does not bind ClpX as other adaptor proteins (Chan *et al.*, 2012), Chan et al. (2014) propose that the YjbH mode of action instead involves disruption of the tertiary Spx protein structure, which exposes the ClpX recognition motif and allows ClpXP-mediated Spx proteolysis.

Interestingly, *yjbH* is itself a Spx-controlled gene and therefore the concentration of YjbH increases upon activation of the regulon (Rochat *et al.*, 2012). This creates a negative-feedback loop in which YjbH levels concomitantly rise with Spx to presumably allow rapid Spx degradation once stress conditions are relieved. This negative loop may also prevent the negative effects of Spx accumulation.

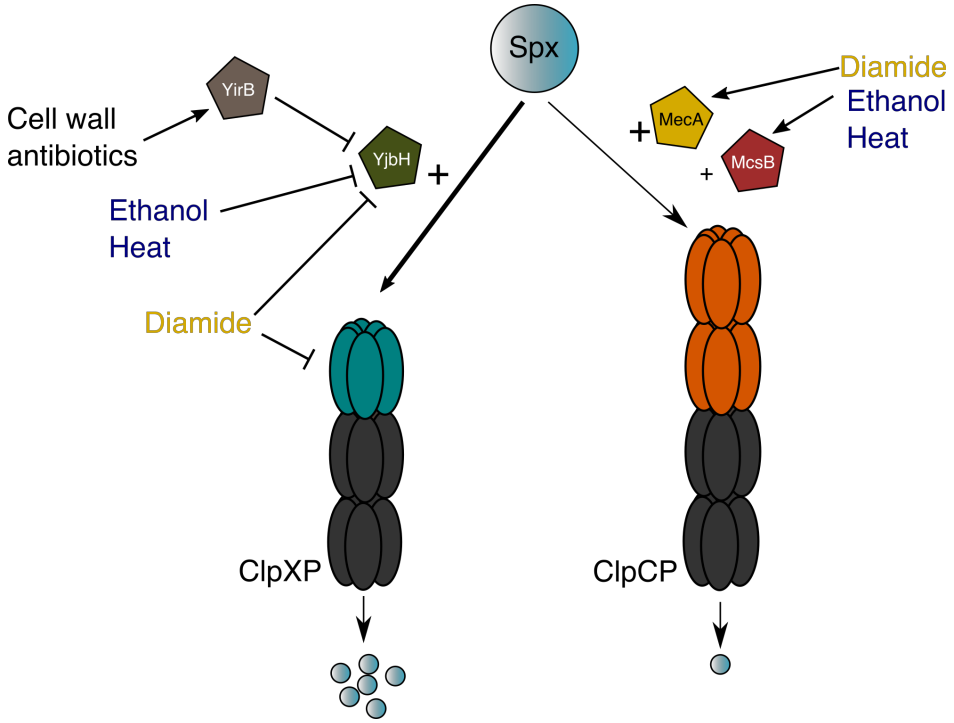

FIG 1.2 MODEL OF SPX PROTEOLYSIS IN B. SUBTILIS.

ClpXP and ClpCP, in a minor extent, are capable of degrading Spx. Protein adaptors and anti-adaptors modulate Spx degradation in response to specific environmental conditions. The activity of the ClpXP protease is also affected by post-translational modifications.

1.5.3 Spx proteolysis through ClpXP is affected by the YirB anti-adaptor protein

YirB is a small protein of 54 amino acids that leads to Spx accumulation when overexpressed. YirB binds YjbH with high affinity, and therefore blocks the Spx-YjbH interaction resulting in Spx accumulation (Kommineni *et al.*, 2011). Although the effect of YirB overexpression was known, its biological role was less clear. Recent studies, however, showed that the YirB anti-adaptor is important for the induction of the Spx regulon in response to cell wall stress (Fig 1.2). Induction of YirB significantly increases the half-life of Spx, and results in strong and long-lasting induction of Spx-controlled genes in response to cell wall-active antibiotics (Rojas-Tapias and Helmann, 2018b).

Elevated expression of *yirB* is detected in vancomycin-treated cells (Rojas-Tapias and Helmann, 2018b), but not diamide- or ethanol-treated cells (Nicolas:2012hc; Rojas-Tapias and Helmann, 2018b). This induction occurs rapidly after treatment with vancomycin and is mediated by the simultaneous action of the CssRS two-component system and the YuxN repressor, whose genes lie downstream and upstream of *yirB*, respectively. YuxN, a TetR-like protein repressor, binds two palindromic DNA sequences located upstream and downstream the *yirB* start transcription site and prevents activation of *yirB* and *yuxN,* possibly through formation of a repression loop. Upon cell wall stress, the CssS histidine kinase phosphorylates CssR, which binds the CssR box located upstream the *yirB* promoter. Interestingly, this CssR box overlaps the YuxN box, and therefore binding of CssR~P to the CssR box leads to disruption of the repression complex; binding of CssR~P thus results in induction of *yirB* (and *yuxN*). The YirB anti-adaptor then binds to and sequesters the YjbH adaptor protein, which results in increased Spx stability. This stabilization is critical for full and timely activation of the Spx regulon in response to cell wall stress (Rojas-Tapias and Helmann, 2018b). Thus, the *yirB* gene is under control of the CssRS two-component system.

Cells lacking CssR are, as expected, unable to induce *yirB* in response to vancomycin treatment (Rojas-Tapias and Helmann, 2018b). Although CssRS responds to cell wall stress (Rojas-Tapias and Helmann, 2018b; Wecke et al., 2011), it was originally reported to be important in the secretion stress response of *B. subtilis* (Darmon et al., 2002; Hyyryläinen et al., 2001). In addition to *yirB*, CssRS also drives its own activation and the expression of two membrane-anchored serine proteases: *htrA* and *htrB*, which are important for quality control of secreted proteins (Darmon et al., 2002; Hyyryläinen et al., 2001). Induction of the CssR regulon is thus observed in response to hypersecretion of soluble proteins such as AmyQ (Hyyryläinen *et al.*, 2001). Activation of CssR-controlled genes is further observed in response to overexpression of lipoproteins (i.e. MntA and YcdH) and soluble proteins (i.e. XynA, Usp45, and Bla) (Marciniak *et al.*, 2012). These results therefore suggest that induction of *yirB* might also occur under conditions of secretion stress, which in

turn would lead to induction of the Spx regulon. In this context, Spx might be important to prevent or alleviate protein aggregation. A global survey to map the *B. subtilis* genome reveals at least four more promoters that may potentially drive the expression of *yirB* (Rochat *et al.*, 2012). This suggests that yet-to-be-determined conditions also might lead to induction of Spx-controlled genes in a *yirB*-dependent fashion.

1.5.4 Spx degradation by the ClpCP protease

In vitro experiments showed that ClpCP and MecA can degrade Spx with high efficiency; however, cells lacking ClpC or MecA do not display increased Spx levels (Nakano et al., 2002). By using a transposon library, we found that deletion of the proteolysis master regulator CtsR resulted in increased Spx proteolysis in cells lacking YjbH or ClpX, thus suggesting an alternative pathway for proteolysis. Further analysis showed, as expected based on *in vitro* data (Nakano et al., 2002), that ClpCP was responsible for Spx degradation. Spx degradation, however, is much faster via ClpXP than through ClpCP *in vivo*. Nevertheless, these observations suggest that ClpCP-dependent Spx proteolysis might be more important under stress conditions that result in inactivation of ClpXP-mediated Spx proteolysis (Fig 1.2). These conditions are observed under disulfide stress, as previously stated. Consistently, Spx proteolysis is enhanced in *ΔclpX* cells when treated with diamide, whereas Spx remains fully stable in diamide-treated *ΔclpC* cells. Given that high Spx levels produce a negative effect on bacterial growth, it is likely that ClpCP-mediated degradation has evolved as a strategy to prevent Spx overaccumulation under oxidative stress conditions.

Protein delivery to ClpCP often requires the activity of specific protein adaptors. In *B. subtilis*, three adaptors have so far been identified: MecA, McsB, and YpbH (Kirstein *et al.*, 2009). In order to identify the adaptor protein required for Spx proteolysis via ClpCP, a series of double mutants simultaneously lacking *clpX* and each of the protein adaptors were constructed. While the double

mutants *ΔclpX ΔmcsB* and *ΔclpX ΔypbH* were easily constructed, a *ΔclpX ΔmecA* double mutant was not obtained. Interestingly, upon deletion of *spx,* the *ΔclpX ΔmecA* double mutant was readily constructed, suggesting that Spx might be toxic for cells lacking functional ClpXP and ClpCP proteolysis. Construction of a *ΔclpC ΔclpX* double mutant was also particularly challenging. In this order of ideas, cells lacking *clpP* should be non-viable as the *ΔclpX ΔmecA* double mutant, however, this is not the case. While transformation experiments revealed that *ΔclpP* cells display a more pleiotropic phenotype than *ΔclpX* cells because of Spx, which is consistent with the existence of an alternative degradation pathway, cells lacking *clpP* are still viable. It is therefore likely that MecA regulates the activity of Spx in other ways. Nevertheless, those results also suggest that MecA is the adaptor protein for Spx degradation via ClpCP, which is consistent with previous biochemical experiments (Fig 1.2).

1.5.5 Arginine phosphorylation affects ClpCP-mediated Spx proteolysis

ClpCP-mediated proteolysis is affected by arginine phosphorylation (Trentini *et al.*, 2016). Arginine phosphorylation is a post-translational modification in which a phosphate group is enzymatically attached to specific arginine residues in proteins. The role of this post-translational modification is thought to resemble the ubiquitination process in Eukaryotes, in which a protein becomes tagged for degradation via the proteasome. In *B. subtilis*, the phosphorylated protein is tagged for degradation via ClpCP. The role of arginine phosphorylation, however, seems to be broader, as significant changes in gene expression are observed upon deletion of the arginine kinase (i.e. McsB) or phosphatase (i.e. YwlE) (Elsholz *et al.*, 2012). A specific case is CtsR, the master regulator of proteolysis, whose activity is dramatically affected by phosphorylation of an arginine residue; in CtsR, phosphorylation of the R63 residue prevents binding of CtsR to DNA, which impairs its ability to repress gene expression (Fuhrmann *et al.*, 2009). This means that conditions that lead to

increased arginine phosphorylation can result in elevated expression of the genes in the CtsR regulon, and in consequence increased proteolysis. Protein arginine phosphorylation is catalyzed by the arginine kinase McsB, whose gene is located in the same operon as *clpC* (Kirstein *et al.*, 2007). The activity of McsB is regulated by McsA, ClpC, and YwlE (Elsholz et al., 2011a; 2011b).

Genetic studies identified YwlE as a potential regulator of Spx stability/activity. Cells lacking YwlE displayed reduced basal levels of P_{trxB}, suggesting that increased arginine phosphorylation reduced Spx activity and/or stability. Analysis of protein stability in unstressed cells showed a decrease in Spx stability in *ΔclpX ΔywlE* compared to *ΔclpX* cells, suggesting that elevated arginine phosphorylation decreases Spx stability probably through increased ClpCP proteolysis. Interestingly, deletion of *mcsB* in a *ΔclpX* strain also reduced Spx stability, which seems to suggest that arginine phosphorylation also stabilizes Spx. Alternatively, deletion of *mcsB* might increase MecA-dependent Spx proteolysis, which might account for these seemingly incoherent results. Further research will allow to elucidate the mechanistic role of arginine phosphorylation on Spx dynamics.

1.6 Posttranslational regulation of Spx

1.6.1 The CXXC disulfide switch

B. subtilis Spx features a redox-sensing switch located at the Spx N-terminus, which consists of the typical CXXC motif (Nakano et al., 2005) (Fig 1.3). Disulfide stress conditions result in oxidation of this motif and formation of a disulfide bond, which positively affects Spx activity (Nakano et al., 2005; Rojas-Tapias and Helmann, 2018a). *In vitro* transcription experiments using purified components and the *trxA* and *trxB* promoters showed the importance of the redox-sensing switch to activate Spx, as purified Spx^{C10A} was dramatically less competent to induce transcription (Nakano et al., 2005). Similarly, while diamide treatment does not lead to significant changes in Spx concentration in *ΔclpX* mutant cells, it greatly increases the activity of a P_{trxB}-*lacZ* fusion (unpublished data). Increased P_{trxB}

activity in absence of changes in Spx concentration therefore reflects post-translational changes. Altogether, these observations demonstrate that modulation of the Spx oxidation state has a profound impact on induction of the Spx regulon

The Spx CXXC motif is positioned distal to the Spx-αCTD binding interface (Newberry *et al.*, 2005), therefore, how formation of the disulfide bond affects gene induction is not entirely clear. Oxidation of the redox-sensing switch might directly affect the nature of the complex formed by Spx and αCTD, which would result in increased activity. Alternatively, oxidation of Spx might affect its interaction with still unknown elements, which may modulate its activity. In support of the first hypothesis, oxidation of the CXXC has been shown to lead to conformational changes in the helix α4, which contains residues involved in transcriptional activation and αCTD/Spx-promoter interaction. The changes occurring at α4 appear to affect the interaction between the αCTD/Spx complex and the promoter, rather than the interaction of Spx with αCTD (Nakano et al., 2010).

Oxidation of the redox-sensing switch, however, is not essential for induction of the Spx regulon. Cells harboring a Spx^{C10A} or $Spx^{C10AC13A}$ mutant protein are still competent to induce the expression of some Spx-controlled genes, in spite of the fact that these Spx proteins are unable to form an intramolecular disulfide bond. Induction of *trxB* is additionally observed in response to diamide in cells harboring the $spx^{C10AC13A}$ allele, suggesting that modulation of protein concentration suffices to activate Spx-controlled genes. Furthermore, a study of the oxidation state of the Spx protein following treatment with cell wall-active antibiotics showed that even though induction of Spx-controlled genes is observed, the protein is present in its reduced state. In this case, the driving force leading to induction of the Spx regulon appears to be the concentration of Spx, rather that its oxidation state.

1.6.2 Is Spx subjected to arginine phosphorylation?

Arginine phosphorylation, as previously discussed, can modulate the activity and/or turnover of specific sets of proteins in *B. subtilis*. Interestingly, Clausen and collaborators found arginine-phosphorylated Spx in the insoluble fraction of *ΔclpP* mutant cells, thus suggesting that this post-translational modification might affect Spx function (Trentini et al., 2016). In the same study, arginine phosphorylation was also observed in MgsR, a Spx homolog protein in *B. subtilis*. Four residues were found to be modified on Spx: R14, R91, R100, and R112 (Fig 1.3), while two residues were observed on MgsR: R17 and R95 (equivalent to R14 and R92 in Spx). Since both proteins were phosphorylated on almost the same residues, this appears to suggest that arginine phosphorylation may be a general property of Spx-like proteins. The position of the residue R112 also provides insight into an additional regulatory role of arginine phosphorylation. This residue is located in the same protein region where the adaptor protein YjbH binds Spx, an inversion of the charge might prevent binding of YjbH to Spx and therefore reduce ClpCP-mediated Spx proteolysis. Since ClpCP proteolysis would then occur at a slower rate, this mechanism would lead to stabilization of a fraction of Spx. We hypothesize that this stable fraction becomes oxidized and drives the expression of Spx-controlled genes in absence of stress, which is consistent with previous observations.

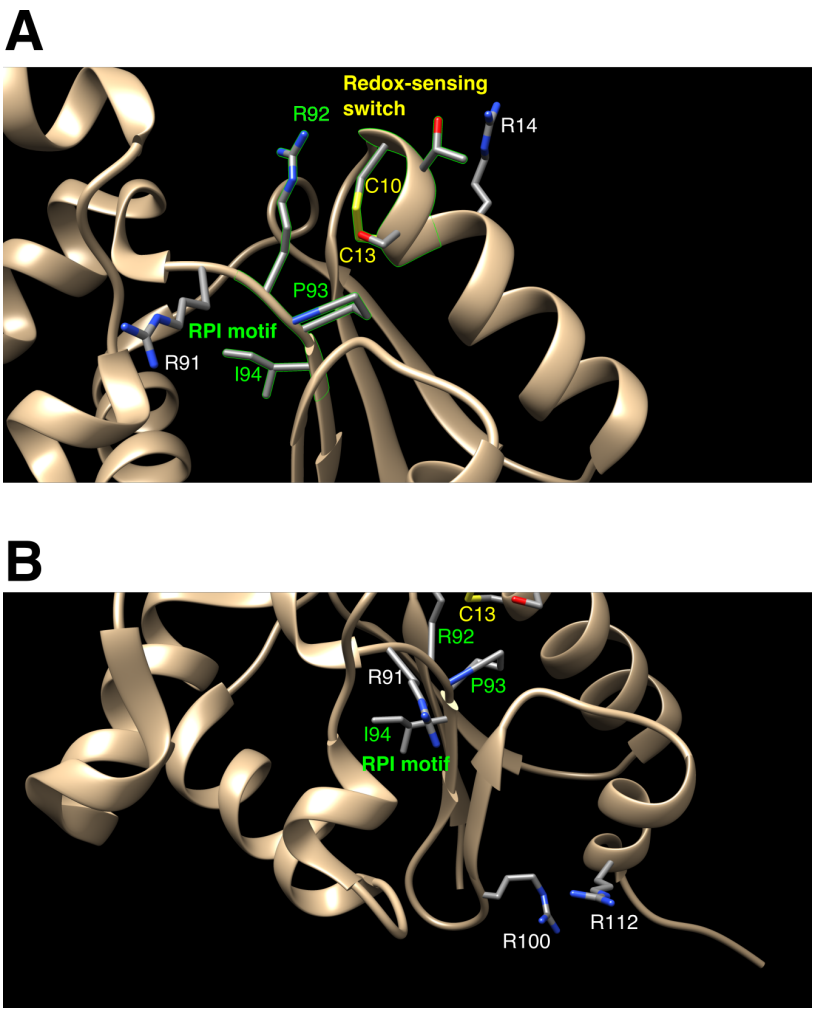

FIG 1.3 POST-TRANSLATIONAL MODIFICATION OF B. SUBTILIS SPX.

A) The redox-sensing switch (yellow residues) and RPI motif (green residues) are illustrated. A) and B) The putatively phosphorylated arginine residues, as described in Trentini et al. (2016), are illustrated in the protein structure.

1.6.3 Is Spx acetylated?

One major difference between the induction of the Spx regulon in response to disulfide and cell wall stress is the differential activation of *yjbC*. Since activation of the *spx* regulon in response to cell wall stress involves the σ^M-dependent activation of the P_{M1} promoter, this stress conditions results in the increased expression of the *yjbC-spx* transcriptional unit. *yjbC* encodes a putative acetyltransferase from the Gcn5-related N-acetyltransferases (GNAT) family of proteins. The GNAT

family of N-acetyl transferases are enzymes that transfer an acetyl group from acetyl coenzyme A to a primary amine of the receptor. Whether YjbC binds and/or modifies Spx is still unclear. The genetic linkage between *yjbC* and *spx*, however, seems to suggest a possible interaction. Additionally, YjbC only appears to be present in *B. subtilis* and closely related species, as it occurs with YirB. Further studies will allow to elucidate the role of YjbC.

1.7 Signal integration: regulation of *B. subtilis* Spx in response to disulfide and cell wall stress

The stress induction of regulons results from discrete changes in signal transduction: transcriptional induction, posttranslational modifications, and/or reduced proteolysis. Induction of Spx involves the simultaneous integration of multiple signals, which allows the cell to respond to different stress conditions using a single transcription factor. Here, we present the current models for Spx regulation in response to disulfide and cell wall stress.

1.7.1 Induction of the Spx regulon in response to diamide (disulfide stress)

Diamide treatment (disulfide stress) results in accumulation and oxidation of Spx, as well as induction of its regulon. However, *spx* mRNA levels under disulfide stress remain unchanged (Rochat *et al.*, 2012; Rojas-Tapias and Helmann, 2018a). The activation of the Spx regulon under this condition therefore relies on post-translational events that permit Spx accumulation and the oxidation of its redox-sensing switch (Fig 1.4). Reduced Spx proteolysis is indeed the key element that allows Spx levels to rapidly change and increase several times in spite of the fact that transcriptional levels are unaffected. Diamide affects Spx proteolysis by interfering with the activity of both the ATP-dependent protease ClpXP (Zhang and Zuber, 2007) and the adaptor protein YjbH (Engman and Wachenfeldt, 2015). The effect of this electrophilic chemical on ClpXP is direct, since diamide treatment results in

oxidation of the ZBD motif in ClpX that determines the activity of the ClpX unfoldase (Zhang and Zuber, 2007). The inactivation of ClpX is not specific, and degradation of even SsrA-tagged proteins is reduced. Other conditions that result in oxidative stress, such as hydrogen peroxide treatment, similarly provoke the oxidation of the ZBD and therefore affect ClpX activity (Zhang and Zuber, 2007). Simultaneously, disulfide stress results in aggregation of the adaptor protein YjbH, which becomes unable to bind Spx, and thus permits Spx accumulation. YjbH inactivation appears to be an indirect effect of diamide treatment as substitution of all seven YjbH cysteine residues does not prevent its aggregation (Engman and Wachenfeldt, 2015). This result therefore implies that other elements within YjbH define its tendency to aggregate under oxidative stress conditions. The combined effect of ClpX and YjbH inactivation consequently results in a significant increase in the amount of Spx protein. Accumulated Spx is then susceptible to direct oxidation by diamide, or other oxidizing agents, at its redox-sensing switch. Upon oxidation of Spx, the protein becomes more competent to modulate gene expression (Nakano et al., 2005; Rojas-Tapias and Helmann, 2018a). The induction of the genes in the Spx regulon in response to diamide stress occurs very rapidly, and after 1 min Spx has already fully accumulated.

1.7.2 Induction of the Spx regulon in response to cell wall stress

Cell wall stress also leads to activation of the Spx regulon, but the molecular events that result in its induction are remarkably different than those observed in response to diamide stress. Treatment with cell wall antibiotics results in up-regulation of the σ^M-regulated P_{M1} promoter which is located upstream *yjbC-spx* (Fig 1.4). *spx* upregulation then leads to an increase in the amount of Spx protein (Rojas-Tapias and Helmann, 2018a). Spx is however still unstable under these conditions, and protein chase assays reveal a Spx half-life of ~ 2 min (Rojas-Tapias and Helmann, 2018b). This increase in *spx* mRNA levels therefore seems to be sufficient to surpass the rapid Spx degradation rate observed

in growing cells, and thus allow for Spx accumulation. Notably, we found that stabilization also plays a significant role in Spx accumulation under cell wall stress conditions. Part of this stabilization is mediated by the anti-adaptor protein YirB, and cells lacking it display reduced Spx turnover (~1 min), and consequently reduced and delayed Spx accumulation (Rojas-Tapias and Helmann, 2018b). Cells lacking YirB, surprisingly, are still able to stabilize Spx in response to vancomycin treatment, thus indicating that unknown stabilization mechanisms are also important under conditions of cell wall stress (Fig 1.4).

In response to cell wall stress, Spx accumulation dynamics are largely dependent on the specific nature of the antibiotic used, as they affect cell wall assembly in distinct manners (Helmann, 2016; Rojas-Tapias and Helmann, 2018a). Using vancomycin, for instance, induction is seen after 5 min of treatment and becomes maximal after ~10 min, while with fosfomycin and cycloserine induction takes ~ 20 min and the peak is reached after ~40 min (Rojas-Tapias and Helmann, 2018a; 2018b). Once the protein accumulates in the cytoplasm, it remains primarily in the reduced state as determined using AMS-alkylation experiments. Further evidence comes from the fact that although a larger concentration of Spx protein is achieved in response to cell wall stress compared with diamide, cell wall stress results in comparatively lower induction of Spx-controlled genes (Rojas-Tapias and Helmann, 2018a). Nevertheless, cells lacking Spx display increased sensitivity towards cell wall antibiotics, suggesting that Spx is critical in the adaptation of *B. subtilis* cells to cell wall stress (Rojas-Tapias and Helmann, 2018a).

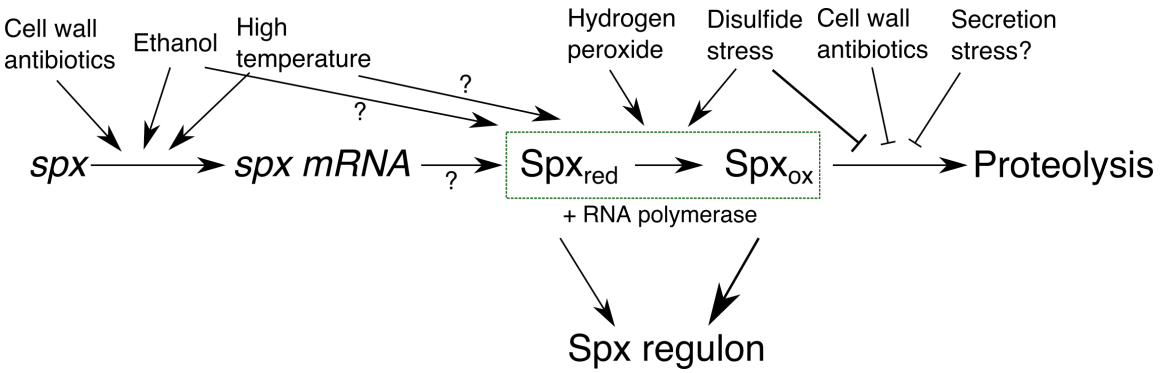

FIG 1.4 SPX REGULATION OCCURS AT MULTIPLE LEVELS IN RESPONSE TO DIFFERENT STRESS CONDITIONS.

Regulation of Spx occurs almost at all levels, suggesting that different signals can result in a similar outcome.

1.7.3 Activation of Spx controlled-genes by heat and ethanol

Although the mechanistic details that result in activation of the Spx regulon in response to both heat and ethanol have not been studied in detail, the existing evidence suggests that these stress conditions might lead to activation of the Spx regulon through transcriptional and post-translational processes (Fig 1.4). Activation of the Spx regulon in response to heat and ethanol, two proteotoxic stresses, has been previously shown. Runde et al. (2014), for instance, showed that heat leads to activation of Spx-controlled genes, and that this activation is important to protect *B. subtilis* cells from heat shock; furthermore, conditional expression of the Spx-controlled gene *trxA*, which encodes a thioredoxin, alleviated the negative effect caused by this stress. Similarly, Engman and von Wachenfeldt (2015) showed that heat and ethanol stress lead to accumulation of Spx, which correlated with aggregation of YjbH.

Our current knowledge on the molecular processes that result in activation of Spx, in addition to current literature, suggests a possible model for activation of Spx in response to these two stress conditions. At the transcriptional level, transcriptomic studies indicate that heat and ethanol are capable of inducing the expression of the *yjbC-spx* operon, which is likely by activation of the σ^B-dependent promoter (Nicolas et al., 2012). We do not rule out a contribution of the P_{M1} promoter, since

σ^M has been shown to be also activated in response to ethanol and other conditions (Thackray and Moir, 2003). And at post-translational levels, aggregation of YjbH at least seem to play a significant role (Engman and Wachenfeldt, 2015). The oxidation of the Spx redox-sensing switch is also likely to play a critical role, since both conditions are likely to lead to oxidative stress.

1.7.4 Induction of Spx by secretion stress

The discovery of YirB as a modulator of Spx proteolysis in *B. subtilis* provided a clear linkage between the secretion stress response and activation of the Spx regulon (Rojas-Tapias and Helmann, 2018b). The expression of *yirB*, as previously mentioned, is driven by the CssRS two-component system, which was first shown to be activated under overexpression of soluble proteins such as the α-amylase (Hyyryläinen et al., 2001; Rojas-Tapias and Helmann, 2018b). This induction thus likely leads to accumulation of Spx protein and activation of the regulon due to reduced Spx proteolysis. Positive correlation between the induction of the CssRS regulon and activation of Spx-controlled genes was observed in transcriptomic studies aimed at studying the expression profile of *B. subtilis* cells upon overexpression of membrane proteins, lipoproteins, and secreted proteins. Results showed that overexpression of two lipoproteins (i.e. MntA and YcdH) and three secreted proteins (i.e. XynA, Usp45, and Bla) led to activation of *cssRS*, *htrA* and/or *htrB*. Activation of Spx-controlled genes was concomitantly observed upon overexpression of YcdH, XynA, and Usp45 (Marciniak et al., 2012). Although this study does not provide direct evidence, it seems to suggest that activation of the Spx regulon also occurs in response to protein secretion stress (Fig 1.4). The role of Spx might be to prevent protein misfolding through induction of the thioredoxin system, for instance, and/or through removal of aggregated proteins by activation of the Clp proteases (Nakano et al., 2003a; Rochat et al., 2012). Further studies will be required to elucidate the importance of Spx under this stress condition.

1.7.5 Induction of the Spx regulon by hydrogen peroxide

The Spx regulon also seems to play an important role under oxidative stress conditions, such as those elicited by hydrogen peroxide (Nicolas et al., 2012). Here, the protein repressor PerR is supposed to play a critical role (Leelakriangsak et al., 2007). Strong upregulation of *spx*, and in a minor extent *yjbC*, and also of Spx-controlled genes was previously observed in *B. subtilis* protoplasts, which display increased levels of reactive oxygen species due to alterations in the electron transport pathway (Kawai et al., 2015). This evidence thus suggests that these conditions might result in activation of the Spx regulon through increased transcription at the P_A promoter (Fig 1.4). Whether these conditions also result in a reduction of Spx proteolysis yet remain unknown. Recent evidence, however, appears to suggest that hydrogen peroxide treatment does not lead to aggregation of YjbH, as observed using a YjbH-GFP translational fusion (Engman and Wachenfeldt, 2015). In the same experiment, ethanol, heat, and diamide led to formation of fluorescent foci, which were indicative of YjbH aggregation. It is therefore likely that transcriptional activation acts as the major driver of Spx accumulation in response to hydrogen peroxide, however, at this point is not possible to rule out additional molecular mechanisms.

1.8 Spx paralogs

The number of Spx-like proteins in representative species within the Firmicutes phylum considerably varies among genera and even species. For simplicity, we define a Spx-like protein as those proteins with at least 30% identity to *Bacillus subtilis* Spx, and/or the presence of the 1) CXXC motif and 2) the RPI motif (Fig 1.3), or 3) the Gly52 residue (Newberry et al., 2005; Zuber, 2004). The large variation in the number of Spx-like proteins among different species appears to suggest that the Spx proteins have evolved to perform specialized functions, as is observed, for instance, in two-component systems or alternative sigma factors (Helmann, 2016; Salazar and Laub, 2015). Different

Spx paralogs can then respond to distinct environmental signals and then activate specific sets of genes. One example of such a specialization is observed in *B. subtilis* cells where one Spx paralog (i.e. Spx) is capable of inducing a specific regulon in response to different stress conditions using multiple activation mechanisms, while the other paralog (i.e. MgsR) is critical for the activation of a subset of σ^B-controlled genes and is itself directly activated by SigB (Engman and Wachenfeldt, 2015; Nakano et al., 2003a; Reder et al., 2008; 2012; Rojas-Tapias and Helmann, 2018a; Rojas-Tapias et al., 2012). Interestingly, both Spx and MgsR seem to discriminate between different holoforms of the RNA polymerase, containing the σ^A or σ^B sigma factors, respectively. The conditions that result in activation of both paralogs, as judged by the expression profile of *trxB* and *mgsR*, are also substantially different; and the set of genes under control of each paralog are different as well (Nakano et al., 2003a; Reder et al., 2008; Rochat et al., 2012). Herein, we will review the Spx paralogs of *B. subtilis* and other Firmicutes.

1.8.1 Spx paralogs in *B. subtilis*

Bacillus subtilis features three Spx homologs: Spx itself, MgsR (46% identity) and YusI (29% identity) (Fig 1.5). MgsR features the characteristic CXXC redox-sensing switch, the Gly52 residue, and the RPI motif, and also acts as a transcription factor modulating gene expression (Reder et al., 2008; 2012). The induction of *mgsR* requires SigB, and this activation is required for MgsR accumulation and activation of MgsR-controlled genes in response to ethanol. MgsR modulates the expression of a subset of σ^B-controlled genes, and cells lacking MgsR display reduced expression of those genes. Furthermore, MgsR features a positive regulatory loop, and therefore also activates its own expression (Reder et al., 2008; 2012). As observed for Spx, MgsR is subjected to proteolysis through ClpXP, and also in a minor extent through ClpCP (Reder et al., 2008; 2012) (Chapter 4). On the other hand, YusI also displays the characteristic CXXC and RPI-like motifs, however, it lacks the

critical Gly52 residue that is required for binding to the RNA polymerase. Whether this protein is also capable of modulating the activity of the RNA polymerase and redirect gene expression remains largely unknown. Experimental evidence from other species, however, seems to suggest that this protein might not act as an Spx ortholog (Kajfasz et al., 2010; Whiteley et al., 2017)

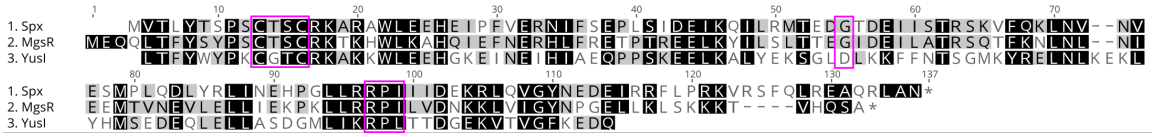

FIG 1.5 SPX PARALOGUES IN B. SUBTILIS

Although all three paralogues contain the characteristics CXXC and RPI motif, only Spx and MgsR possess the critical glycine residue located at Spx^{G52} or $MgsR^{G55}$.

1.8.2 Spx paralogs in *Bacillus*

In the same way as in *B. subtilis*, most of the *Bacillus* species contain two Spx paralogs featuring the three characteristic protein motifs mentioned above. While an Spx paralog with a similarity above 80% to *B. subtilis* Spx was observed in all species, the second Spx paralog displayed similarities ranging between 45% and 61%. Alignment and clustering analysis of the Spx paralogues with > 30% similarity in *B. subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *B. cereus*, *B. thuringiensis*, and *B. anthracis* grouped the Spx paralogs into two different clades (Fig 1.6A). One big clade was formed by those proteins with >56% similarity to *B. subtilis* Spx, and the other clade was formed by those proteins related to *B. subtilis* MgsR. This largest clade was divided into two more clades: one that grouped the homologs related to *B. subtilis* Spx, and another that clustered *B. anthracis* SpxA2 related proteins (Fig 1.7).

Within the *B. subtilis* Spx-containing clade, the strains in the *B. cereus* group (i.e. *B. cereus*, *B. thruringiensis*, and *B. anthracis*) were clustered together, while the strains in the *B. subtilis* complex

(i.e. *B. subtilis* and *B. amyloliquefaciens*) as well as *B. megaterium* formed another cluster (Fig 1.7). With respect to the SpxA2 cluster, it primarily contained strains from the *B. cereus* group; interestingly, a plasmid-encoded Spx homologue of *B. megaterium* also grouped in this cluster. Since *B. subtilis* Spx and *B. anthracis* SpxA1, as well as *B. anthracis* SpxA2, interact with the RNA polymerase harboring the σ^A sigma factor, we hypothesize a similar function for those orthologs present in the other related *Bacillus* species. The other clade clustered *B. subtilis* MgsR with Spx homologues of *B. megaterium* and *B. amyloliquefaciens*. Since MgsR acts on the σ^B-containing RNA polymerase (Reder *et al.*, 2008), it is likely that this homolog displays a similar function in both *B. megaterium* and *B. amyloliquefaciens*. Based on functional and clustering analysis, three Spx homologs were thus identified within the *Bacillus* genus: SpxA1 (the closest homologue to *B. subtilis* Spx), SpxA2 (found in *B. anthracis* and other members of the *B. cereus* group, identity ~60% to *B. subtilis* Spx), and MgsR (found in *B. subtilis* and related species, similarity ~50% to *B. subtilis* Spx). Since the plasmid-encoded Spx homologue in *B. megaterium* grouped with *B. anthracis* SpxA2, this species, unlike the other *Bacillus* species studied here, harbors all three Spx homologues: SpxA1, SpxA2, and MgsR.

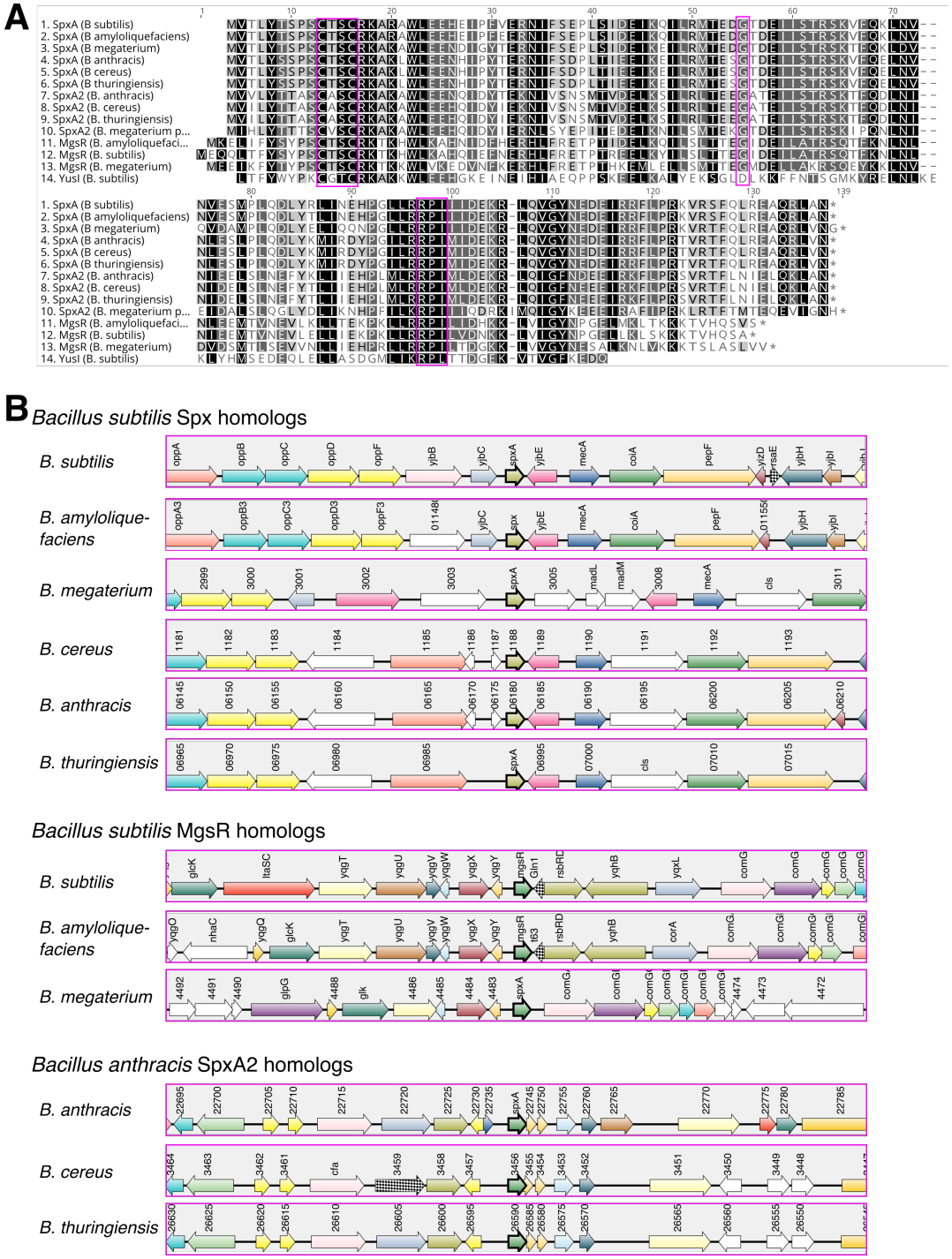

FIG 1.6 ANALYSIS OF THE SPX ORTHOLOGS IN REPRESENTATIVE BACILLUS SPECIES

A) Alignment of Spx homologs in representative *Bacillus* species. B) The genetic context of the different Spx homologs in different species.

Analysis of their genetic vicinity further confirms the presence of three different Spx homologs in *Bacillus*, as they display unique genetic contexts (Fig 1.7). In all species *spxA1* is located downstream the *oppABCDF* operon and upstream *mecA* and *coiA*. The *yjbC* putative acetyltransferase gene is only present in *B. subtilis* and *B. amyloliquefaciens*. As for *mgsR*, its genetic context is almost identical between *B. subtilis* and *B. amyloliquefaciens*, and somewhat less similar for *B. megaterium*. In all cases, nevertheless, the *comGA*, *comGE*, *glcK*, and *yqgT* genes, for example, are located in the vicinity. With respect to *spxA2*, the genetic neighborhood is almost identical among *B. anthracis*, *B. thuringiensis*, and *B. cereus*: two transcriptional regulators are located upstream *spxA2*, a small *yirB*-like-size protein downstream, and a serine transporter further downstream. In regard to the plasmid-encoded *spxA2*, this gene is surrounded by genes involved in transposition (data not shown).

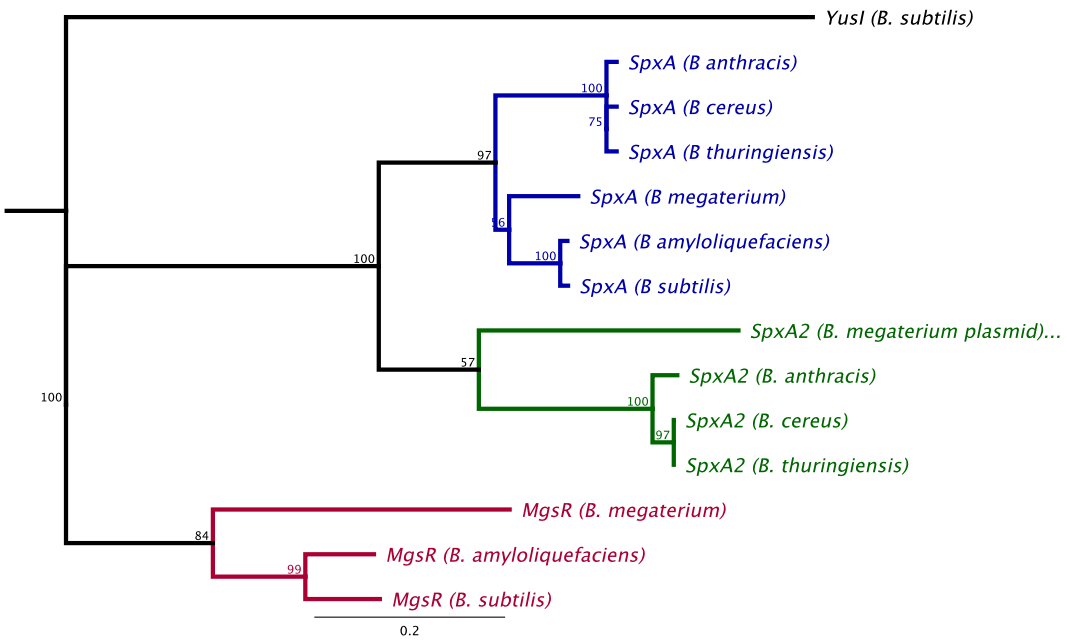

FIG 1.7 PHYLOGENETIC ANALYSIS OF THE SPX ORTHOLOGS IN BACILLUS

Representative genomes of *B. subtilis, B amyloliquefaciens, B. megaterium, B. cereus, B. thruringiensis,* and *B. anthracis* were chosen for the analysis. The YusI protein, the most distant Spx paralogue in *B. subtilis* which lacks the G52 residue, was chosen as outgroup for the phylogenetic tree. This tree was constructed using the neighbor-joining method and the Jukes-

Cantor genetic distance model. A consensus tree was generated after resampling using Bootstrap with 100 replicates. Both alignment and tree were performed in the Geneious software. Colors were used to illustrate the clustering of the different Spx homologs along different species.

1.8.3 Spx paralogs in other Firmicutes

The Spx protein and its homologs are also present in other Firmicutes, including several human pathogens. While three Spx-like proteins are present in *Bacillus*, this number considerably varies in other Firmicutes. *S. aureus* and *S epidermidis,* for example, contain only one Spx protein with >30% identity to *B. subtilis* Spx. These Spx homologs contains all the characteristic features of *B. subtilis* Spx, and are competent to induce gene expression (Pamp *et al.*, 2006; Wang *et al.*, 2010). In comparison, *Lactococcus lactis* and *L. garvieae* possess six and eleven Spx-like proteins. In *L. lactis* only four of them contain identically all three typical features, while the other two homologs lack the typical redox-sensing switch and instead harbor the CSSS and NQSA motifs (Fig 1.8). In *L. garvieae,* seven Spx-like proteins do not contain this protein motif. These non-typical Spx motifs are also observed in other species such as *Enterococcus faecium* and *Lactobacillus acidophilus,* for instance. Recent investigations have shown that formation of the disulfide switch is not required for activation of the Spx regulon in *B. subtilis* (Gaballa and Helmann, 2002; Rochat *et al.*, 2012; Rojas-Tapias and Helmann, 2018a), which seems to imply that this motif might be or not dispensable under some environmental conditions. Homology modeling indeed shows that those Spx homologues display a similar tertiary structure to the canonical Spx, which suggest that they might be competent to bind RNA polymerase and modulate gene expression (Fig 1.8). Some other important Firmicutes such as *Listeria monocytogenes* and *Staphylococcus aureus* contain one, while *Streptococcus pneumonia, Streptococcus pyogenes,* and *Lactobacillus acidophilus* contain two Spx proteins. Spx proteins are absent from representative *Clostridium* species.

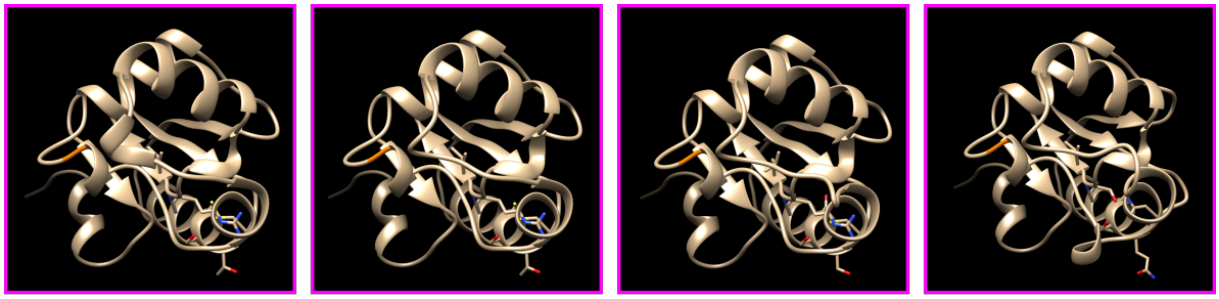

FIG 1.8 HOMOLOGY MODELLING OF SPX HOMOLOGS IN B. SUBTILIS AND L. LACTIS

The tertiary structures of *B. subtilis* Spx, and *L. lactis* SpxA1, SpxA5, and SpxA6 are depicted. The residues in the redox-sensing switch and the RPI motif are displayed. The Gly52 residue is shown in orange.

1.8.3.1 Spx paralogs in L. monocytogenes and S. aureus

Both *L. monocytogenes* and *S. aureus* contain only one Spx homolog with >30% identity and the characteristic Gly52 residue (Fig 1.9A). A second Spx homolog that does not meet the aforementioned criteria is, however, found in both species (Fig 1.9C). In *L. monocytogenes*, this second Spx homolog itself seems to be unimportant for pathogenesis and oxidative stress; however, cells lacking SpxA1 and SpxA2 are more sensitive than *ΔspxA1* to oxidative stress (Whiteley et al., 2017). The Spx homologs in *L. monocytogenes, S. aureus,* and *B. subtilis* appear to share a common origin (Fig 1.9C). Clustering analysis, for instance, revealed that the Spx proteins in *L. monocytogenes* and *S. aureus* are more closely related to *B. subtilis* Spx than other Spx homologs. Further evidence emerges from the genetic context in these three species, as the genes that surround *spx* are almost identical (Fig 1.9B).

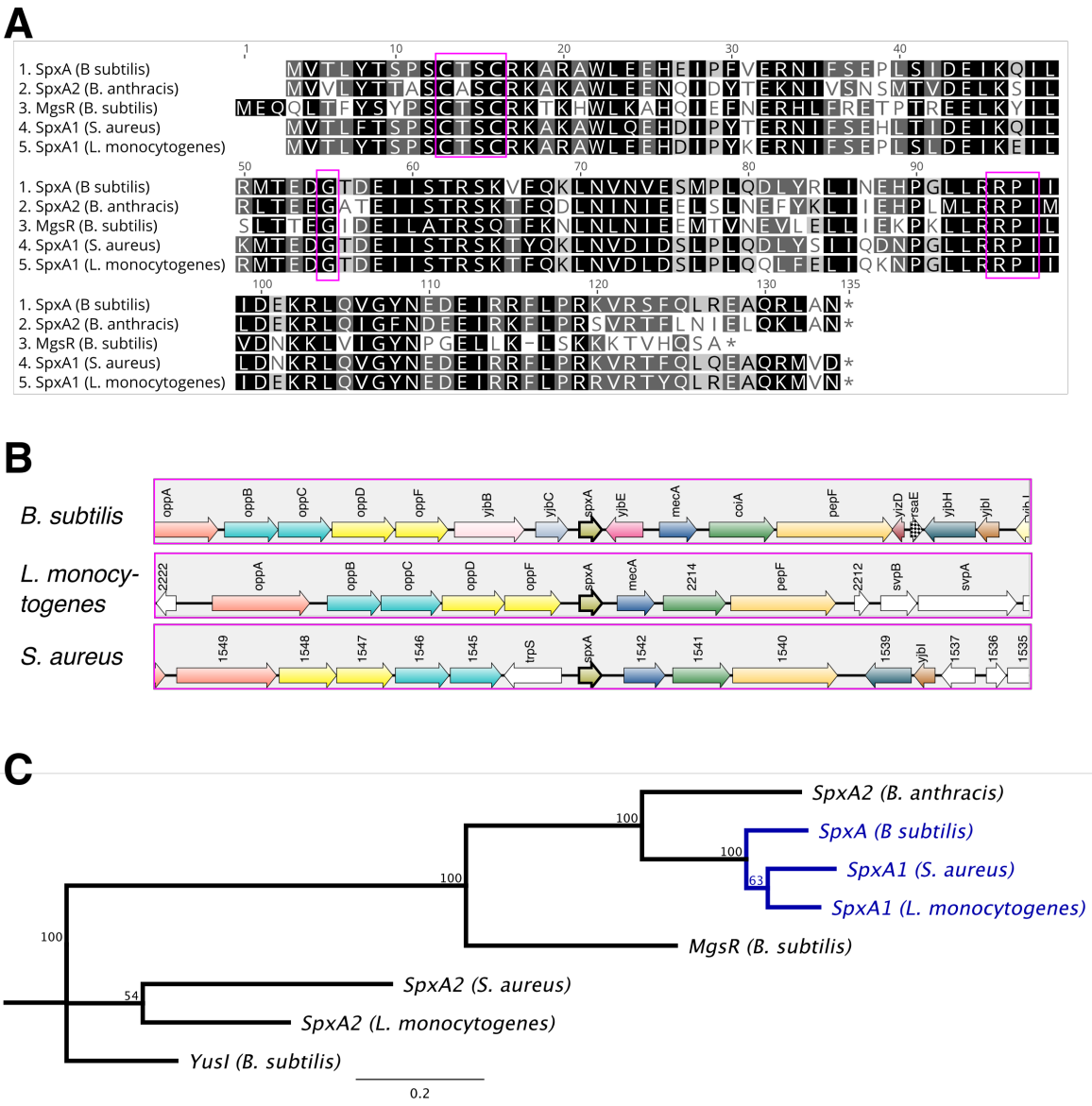

FIG 1.9 SPX PARALOGUES IN S. AUREUS AND L. MONOCYTOGENES

A) Alignment of *S. aureus and L. monocytogenes* SpxA1 with *Bacillus* Spx orthologs. The typical features are highlighted on the alignment. B) Genetic context of *spxA1* in *B. subtilis*, *L. monocytogenes,* and *S. aureus*. C) Phylogenetic analysis of the Spx paralogues. The *B. subtilis* Spx, MgsR, and YusI proteins, as well as *B. anthracis* SpxA2, were included for reference. *B. subtilis* YusI protein was chosen as outgroup for the phylogenetic tree. This tree was constructed using the neighbor-joining method and the Jukes-Cantor genetic distance model. A consensus tree was generated after resampling using Bootstrap with 100 replicates. Both alignment and tree were performed in the Geneious software. Colors were used to illustrate the clustering of the different Spx homologs along different species.

1.8.3.2 Spx paralogs in L. lactis

Lactobacillus lactis appears to be unique among other Firmicutes, as six Spx-like proteins are found in its genome (Fig 1.10A). A seventh paralog is present, however, displays only 21% identity with *B. subtilis* Spx and lacks the G52 residue. As mentioned above, the CXXC and RPI motifs are not present in all *L. lactis* Spx paralogs, including SpxA5 and SpxA6. The function of SpxA6 (formerly SpxB), which does not contain any of those motifs, however, has been demonstrated *in vivo* (Veiga et al., 2007). The genetic context of the Spx paralogs in *L. lactis* is also different in comparison with the genetic context of both *spx* and *mgsR* in *B. subtilis* or *spxA2* in *B. anthracis* (Fig 1.10B). Phylogenetic analysis of the *L. lactis* Spx paralogues, including SpxA7, shows that all SpxA1 to SpxA6 paralogues are more closely related to *B. subtilis* Spx than *B. subtilis* MgsR (Fig 1.10C). It also shows that SpxA3, SpxA5 and SpxA6 might share a common origin. As expected, *L. lactis* SpxA7 did not group with *B. subtilis* Spx, and instead it did with *B. subtilis* YusI.

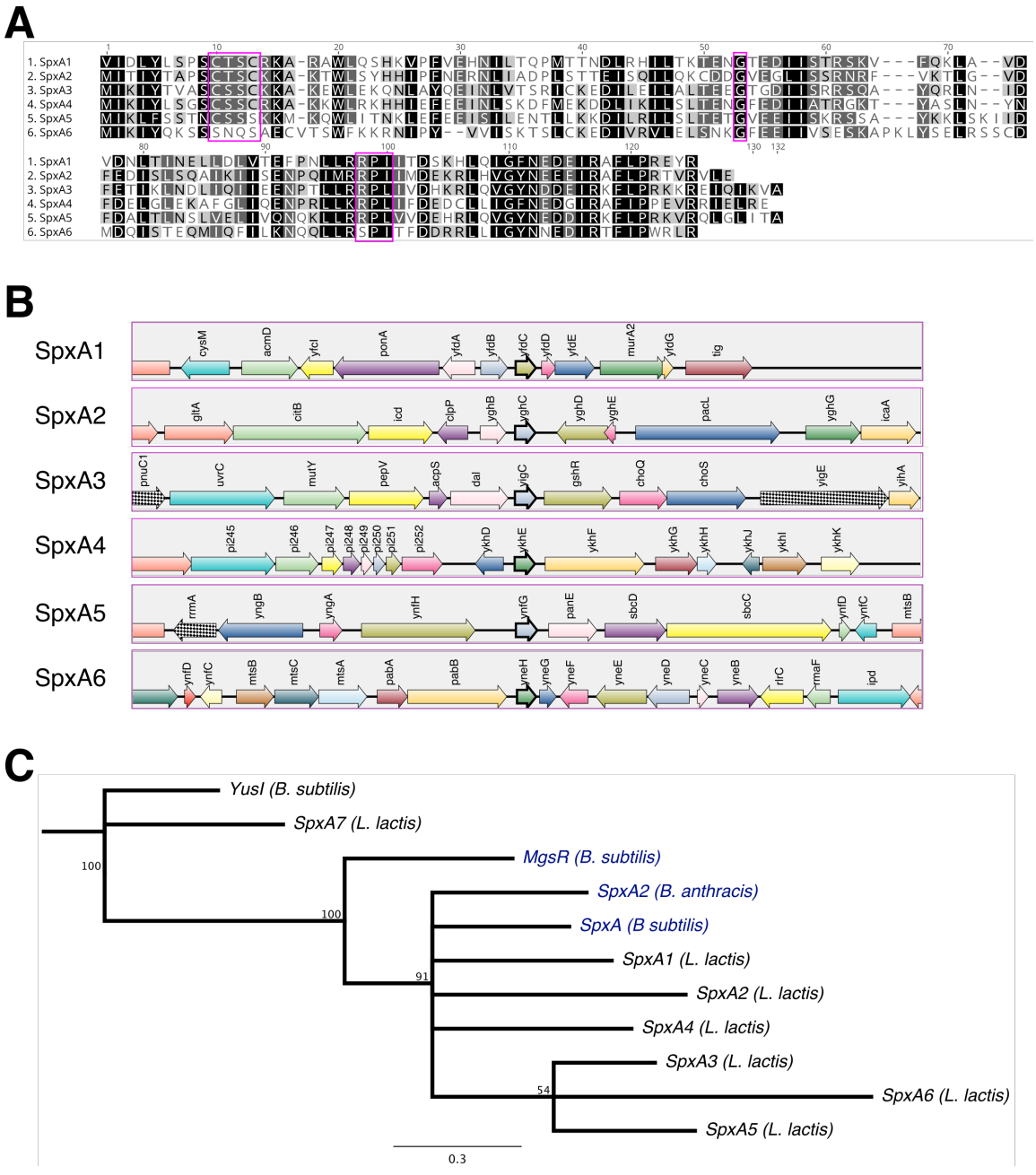

FIG 1.10 SPX PARALOGUES IN LACTOCOCCUS LACTIS

A) Alignment of *L. lactis* Spx paralogs. The typical features are highlighted on the alignment. B) Genetic context of the different *spx* genes. C) Phylogenetic analysis of the Spx paralogues. The *B. subtilis* Spx, MgsR, and YusI proteins, as well as *B. anthracis* SpxA2, were included for reference. *B. subtilis* YusI protein was chosen as outgroup for the phylogenetic tree. This tree was constructed using the neighbor-joining method and the Jukes-Cantor genetic distance model. A consensus tree was

generated after resampling using Bootstrap with 100 replicates. Both alignment and tree were performed in the Geneious software. Colors were used to illustrate the clustering of the different Spx homologs along different species.

1.8.3.3 Spx paralogs in *Streptococcus*

Streptococcus strains contain two Spx paralogs (Fig 1.11). In *S. mutans* and *S.* pneumoniae, the first paralog contains all typical motifs observed in *B. subtilis* Spx, while the second homolog contains a SPI motif instead of the characteristic RPI motif (Fig 1.11A). The *spxA1* and *spxA2* genes are each located in similar genetic context in various *Streptococcus* species, although *S. pneumoniae* seems to be somewhat distinct (Fig 1.11B). Nevertheless, phylogenetic analysis of the Spx paralogues in *Streptococcus* species shows clustering of the SpxA1 homologs, suggesting a common origin (Fig 1.11C). The SpxA1 homologs were further grouped in a clade that also contained *B. subtilis* Spx and *B. anthracis* SpxA2. This clustering suggests that the *Streptococcus* SpxA1 is more closely related to Bacillus Spx and SpxA2 proteins than *Streptococcus* SpxA2. Despite this closeness, the genetic contexts are dissimilar. *Streptococcus* SpxA2 was grouped in a different branch, and seems to represent a distinct Spx homolog. Its genetic context consist of proteins involved in DNA repair.

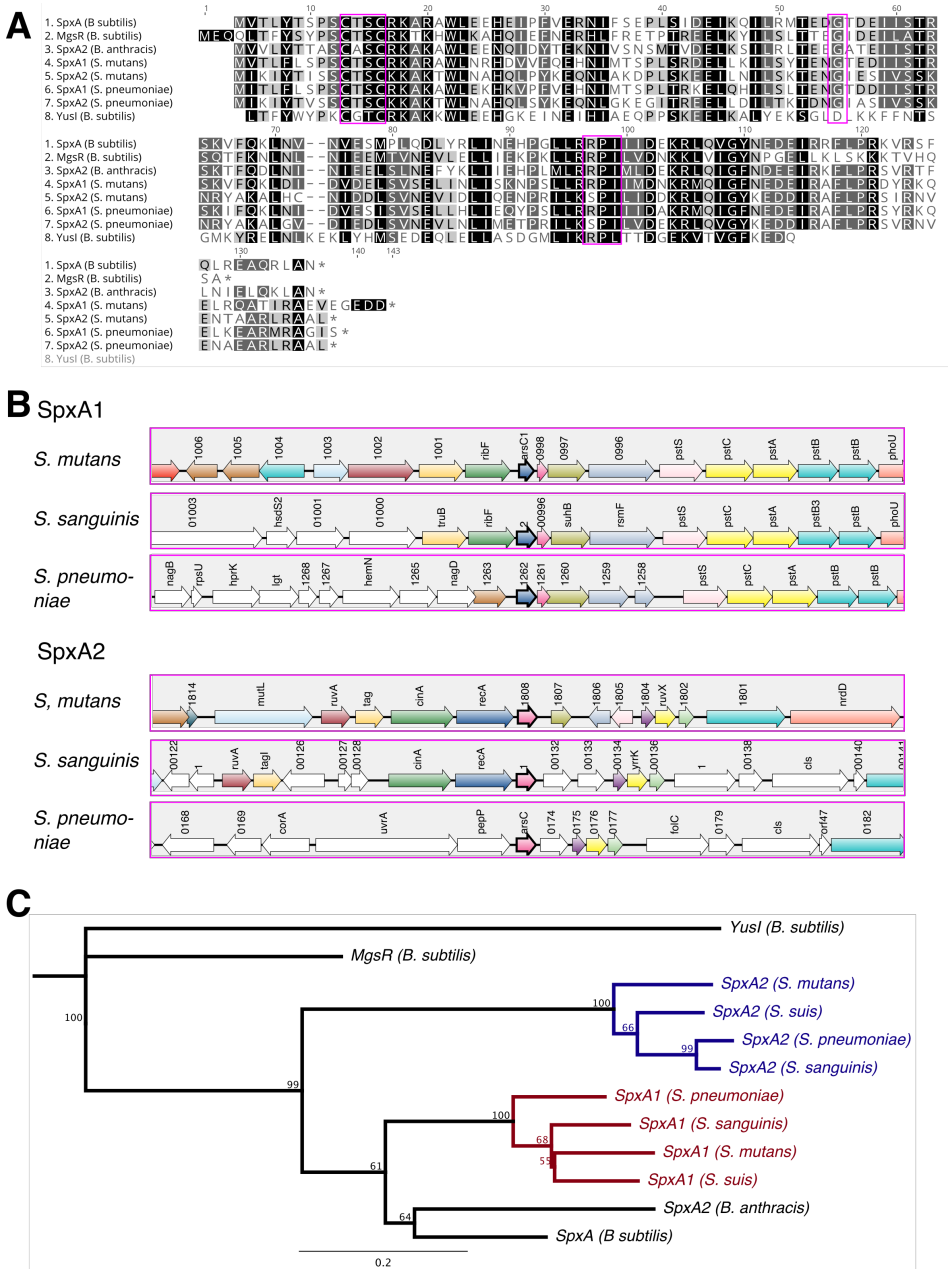

FIG 1.11 SPX PARALOGUES IN STREPTOCOCCUS

A) Alignment of *S. mutans* and *S. pneumoniae* Spx paralogs. The typical features are highlighted on the alignment. B) Genetic context of the different *spx* genes in *S. mutans, S. sanguinis*, and *S. pneumoniae*. C) Phylogenetic analysis of the Spx paralogues in *S. mutans*, *S. sanguinis*, *S. suis*, and *S. pneumoniae*. The B. subtilis Spx, MgsR, and YusI proteins, as well as *B. anthracis* SpxA2, were included for reference. *B. subtilis* YusI protein was chosen as outgroup for the phylogenetic tree. This tree was constructed using the neighbor-joining method and the Jukes-Cantor genetic distance model. A consensus tree

was generated after resampling using Bootstrap with 100 replicates. Both alignment and tree were performed in the Geneious software. Colors were used to illustrate the clustering of the different Spx homologs along different species.

1.9 Regulation and function of Spx in other Firmicutes

The study of Spx in response to diamide provided the foundations of our understanding of the regulatory mechanisms that drive the activation of the Spx regulon in *Bacillus subtilis*. Work of our lab, however, showed that other stress conditions can also activate the regulon, and that the molecular mechanisms associated are distinct. The oxidation of the redox-sensing switch, for example, which was considered virtually essential for the activation of the regulon, has been shown to be dispensable. MgsR, another Spx homologue in *B. subtilis* is even induced by other stress conditions, interacts with a different holoform of the RNA polymerase, and activates a different set of genes. The case for other species seems to be even more complex, as some of them possess a larger number of Spx paralogs. Further, some of those paralogs lack those signatures that we and others considered to be critical to define Spx-like proteins. Spx proteins are important, not only for oxidative stress, but also for virulence, biofilm formation, and adaptation to cell wall stress. Altogether, the current evidence suggests that the regulation and role of Spx proteins is more complex and broader that previously anticipated. Here, we describe our current knowledge on the regulation and function of Spx proteins in representative Firmicutes

1.9.1 *Bacillus anthracis*

Bacillus anthracis, the etiological agent of anthrax, contains two Spx paralogs, named SpxA1 and SpxA2. SpxA1 and SpxA2 display 81% and 61% identity with *B. subtilis* Spx, respectively. Both Spx paralogues contain the characteristic protein motifs. The *spxA1* gene is embedded in a genetic context with syntenic similarity to Bs *spx*, while *spxA2* has no synteny with *B. subtilis*. *spxA2* is the first

gene in a five genes operon along with three hypothetical genes and *saiR,* a gene that encodes a protein repressor. Those five genes are thought to constitute an operon, as deletion of *saiR* resulted in increased expression of *spxA2*, two hypothical genes downstream *spxA2*, and one downstream *saiR* (Nakano et al., 2014)

Cells lacking SaiR displayed increased expression of *spxA2,* thus suggesting that SaiR acts as a repressor of *spxA2*. The direct repression of SaiR on *spxA2* was shown by integration of both a P_{spxA2}-*lacZ* fusion and a IPTG-inducible *saiR* into *B. subtilis*, restriction enzyme protection assays, as well as *in vitro* transcription assays. Repression of *spxA2* relies on SaiR binding two dyad-symmetry motifs located just upstream the start-transcription site (position -23 to -1) and at position +139 to +157. Disruption of any of those boxes abolished SaiR-mediated repression. SaiR belongs to the Rrf2 family of transcriptional regulators, which commonly display a C-terminal domain that function as a sensor for different environmental cues. Indeed, SaiR contains a critical Cys96 residue, which is required for DNA binding as judged using a strain harboring a *spxA2*^{C96S} allele and hydrogen peroxide sensitivity assays. Activation of the *spxA2* gene in *B. subtilis* was observed in response to NaClO, H_2O_2, and diamide; however, this induction was not only mediated by SaiR, but also Bs Spx. The mechanisms by which SaiR regulates *spxA2* seem to involve a direct action of the oxidizing agent on the SaiR-ligand interaction.

1.9.2 *Staphylococcus aureus*

S. aureus, an opportunistic human pathogen, possess only one Spx protein, which displays high similarity with Bs *spx* in terms of protein sequence (80% similarity) and it is located within a similar genetic context. *S. aureus* Spx contains the typical CXXC and RPI motifs, as well as the Gly52 residue. In *S. aureus*, the *spx* gene is located upstream *mecA* and *coiA*, and downstream *trpS* and the *opp* operon.

In the same way as *B. subtilis*, Spx concentration is low in unstressed cells, but highly abundant in cells lacking *yjbH, clpX* or *clpP* (Engman et al., 2012; Pamp et al., 2006). In *S. aureus*, YjbH also acts as an adaptor for ClpXP proteolysis (Engman et al., 2012; Pamp et al., 2006). Spx expression also appears to be constitutive, as high temperature, 1 M NaCl, and 2 mM diamide had no effect on *spx* transcription; cells lacking either *clpX* and *clpP*, however, display reduced levels of *spx* mRNA, suggesting that in *S. aureus spx* might be subjected to autorepression. (Pamp *et al.*, 2006). As expected, induction of *trxB* is Spx-dependent and strongly induced in response to heat and diamide stress, since no changes are observed at transcriptional levels it is likely that post-translational regulation is involved. The *msrA1* and *trfA* (a *mecA* homologue) genes are also induced in a Spx-dependent fashion, and their induction has been shown in response to cell wall antibiotics (Jousselin et al., 2013; Renzoni et al., 2011).

Inactivation of *spx* has a negative effect on *S. aureus* growth rate, and resistance against diamide, high and low temperatures, high NaCl, and high osmolarity (Pamp *et al.*, 2006). Spx is also likely to be important for resistance against cell wall antibiotics, as *ΔyjbH* cells display increased oxacillin resistance (Gohring *et al.*, 2011). Spx also appears to affect biofilm formation via indirect activation of the *icaABCD* genes, which are involved in the synthesis of the polysaccharide intercellular adhesin (Pamp *et al.*, 2006). An effect of Spx on biofilm formation has also been observed in the related species *S. epidermidis*, but in this case Spx stimulates its synthesis (Wang *et al.*, 2010). Taken together, Spx displays a regulatory mechanism that resembles Bs, and plays a remarkable role in the growth, development, and general stress response of *S. aureus.*

1.9.3 *Listeria monocytogenes*

Listeria monocytogenes, the causal agent of the infection *listeriosis*, like *S. aureus*, contains only one Spx protein with >30% identity with Bs Spx (i.e. SpxA1). In the *L. monocytogenes* genome,

however, another Spx homologue is present, which shares 25% identity to Bs Spx (i.e. SpxA2) and has been previously studied (Whiteley *et al.*, 2017). One major difference between those two Spx homologues lies on the fact that SpxA2 contains a Pro residue instead of the critical Gly at position 52. How this proline residue affects the interaction of a Spx protein with the α-CTD of RNA polymerase is unknown. Nevertheless, unlike SpxA1, SpxA2 is unimportant to protect cells against diamide, hydrogen peroxide, and sodium nitroprusside. An additive effect, however, is observed for both SpxA1 and SpxA2 in response to these oxidative agents (Whiteley *et al.*, 2017). SpxA2 also appears not to be required for intracellular growth and cell-to-cell spread in *in vitro* plaque assays; *spxA1* knockdown, conversely, causes a significative reduction in plaque area. Cells lacking *spxA2* yet present a growth rate defect compared with wild-type (Whiteley *et al.*, 2017).

In contrast to most species harboring Spx-like proteins, the SpxA1 protein in *L. monocytogenes* is essential (Borezee *et al.*, 2000; Reniere *et al.*, 2016). Its essentiality, however, can be reversed under anaerobic conditions, indicating that SpxA1 plays a critical role in defending *L. monocytogenes* from the toxicity of reactive oxygen species (Whiteley *et al.*, 2017). In agreement with this result, Reniere et al. (2016) observed that knockdown *spxA1* strains exhibited reduced listeriolysin O (LLO) levels and survival in plaque assays; since *hyl* overexpression only partially complemented the *spxA1* knockdown strain, and a similar phenotype was observed for *ohrR* inactivation, the authors conclude that SpxA1 must be important to survive the harsh conditions found in the vacuole (Reniere *et al.*, 2016). *ΔspxA1* cells, however, are still virulent and able to successfully replicate in the host cytosol (Whiteley *et al.*, 2017).

spxA1 is located upstream *mecA* and downstream the *oppABCDF* operon. The *spxA1* gene is predicted to have two promoters, which are active during both exponential and stationary growth (Borezee *et al.*, 2000; Wurtzel *et al.*, 2012). During both growth phases three transcripts of ~0.5, ~0.6, and ~1.5 kb are detected as determined by northern blot; this latter transcript is the result of co-transcription with *mecA* (Borezee *et al.*, 2000). Either one of those promoters or another promoter

must be inducible within the host cell, as significant upregulation of *spxA1* is observed upon infection compared to cells growing on BHI medium (Chatterjee *et al.*, 2006). None of these three *spxA1* promoters is, however, induced in response to a 37ºC to 42ºC temperature upshift (Borezee *et al.*, 2000). In SpxA1, the CXXC motif seems to play an important role but it is dispensable, as cells harboring an AXXA motif instead are able to growth under aerobic conditions. These cells, however, display reduced growth rate under aerobic conditions in flasks and increased sensitivity against diamide (Whiteley *et al.*, 2017). The capacity of both reduced and oxidized Spx to activate gene expression might be particularly important during pathogenesis, as cells have to transition from the oxidizing phagosome to the reducing cytosol (Myers *et al.*, 2003). During growth in the phagosome, Spx oxidation might drive the activation of the regulon and protect cells against the oxidizing environment, whereas in the cytosol the upregulation of SpxA1-controlled genes might be driven by increased *spxA1* transcription (Chatterjee *et al.*, 2006). Deletion of *yjbH* also had a significant effect on *L. monocytogenes* virulence, which might be due to SpxA1 accumulation (Whiteley *et al.*, 2017). Although speculative, it is likely that YjbH also plays a critical role in the regulation of Spx, as it is observed in *S. aureus* and *B. subtilis* (Engman et al., 2012; Kommineni et al., 2011; Larsson et al., 2007).

1.9.4 *Lactococcus lactis*

Lactococcus lactis, a bacteria extensively used in the dairy industry, contains six Spx paralogs, two of which have been previously shown to modulate gene expression and physically interact with RNA polymerase: TrmA and SpxB (Veiga *et al.*, 2007). As an attempt to unify the nomenclature, we propose to rename the different *L. lactis* Spx paralogues based on their similarity to Bs Spx, as SpxA1 (60% identity), SpxA2 (formerly TrmA, 53% identity), SpxA3 (50% identity), SpxA4 (46% identity), SpxA5 (45% identity), and SpxA6 (formerly SpxB, 30% identity). SpxA1, SpxA2, SpxA3, and SpxA4

contain all the typical features of *B. subtilis* Spx, while both SpxA5 and SpxA6 lack the typical redox-active center. SpxA5 contains a CSSS motif and therefore is not subjected to canonical redox control. SpxA6, by contrast contains the largely unusual amino acid signature NQSA at the site of the canonical CXXC motif. Further, SpxA6 does not contains the RPI motif but instead possesses a SPI motif.

The *spxA2* gene (formerly *trmA*) was identified as the locus for transposon insertions that suppressed the temperature-sensitive phenotype of *ΔclpP* (Frees *et al.*, 2001) *and ΔrecA* (Duwat *et al.*, 1999) mutants. Inactivation of *spxA2* results in increased proteolysis and thus confers thermoresistance; interestingly, this effect is more prominent under anaerobiosis. SpxA2 thus appears to function as a negative element for transcription of genes involved in proteolysis. Interestingly, *spxA2* mRNA levels do not change in response to multiple conditions (Frees *et al.*, 2001), thus suggesting that SpxA2 dynamics must involve post-translational regulation. Deletion of *spxA2* fully abolishes the increased expression of genes within the CtsR and HtrA regulons observed in *ΔclpP* cells (Frees *et al.*, 2001), thus suggesting positive control. These seemingly contradictory observations are likely the result of yet unknown genetic elements. *spxA2* inactivation also increases the resistance of *L. lactis* cells against tellurite (Turner *et al.*, 2007), an agent that leads to oxidative stress, and puromycin (Frees *et al.*, 2001).

Resistance against lysozyme is conferred by overexpression of another paralogue: *spxA6*, the most divergent Spx homologue in *L. lactis*. SpxA6-dependent upregulation of *oatA*, a gene that encodes a peptidoglycan O-acetylase (i.e. OatA), is largely responsible for this phenotype. Interestingly, *spxA6* itself is under control of CesRS, a two-component regulatory system that responds to cell wall stress (Veiga *et al.*, 2007). Since SpxA6 does not possess a redox-active center, activation of SpxA6-controlled genes is likely due to *spxA6* upregulation and maybe SpxA6 stabilization. Moreover, downstream *spxA6* lies a putative regulator that belongs to the ArsD family of protein repressors, which might also affect *spxA6* transcription as observed in the *arsRDABC* operon of *E. coli* (Li et al., 2002; Wu and Rosen, 1993). This system, therefore, is reminiscent of the cell wall

antibiotic-mediated induction of the Spx regulon in *B. subtilis*. The genes that appear to be under SpxA6 and Bs Spx control are, however, distinct.

1.9.5 *Streptococcus mutans*

S. mutans, a major etiological agent of dental caries*,* contains two Spx homologues: SpxA1 (61% identity) and SpxA2 (44% identity). Both Spx homologues contain the CXXC motif and critical Gly52 residue, however, the RPI motif is only found in SpxA1. SpxA2, instead, contains a SPI motif. There is a third Spx homologue in *S. mutans*; however, it lacks the Gly52 residue and instead has a Glu residue. The importance of Spx^{G52} has also been shown in *S. mutans* (Kajfasz *et al.*, 2015). Deletion of this third homologue, as opposed to deletion of *spxA1* or *spxA2*, has no impact on the phenotype of a *clpP* or *clpX* knockout mutant (Kajfasz *et al.*, 2009); this third gene thus does not seem to encode a true Spx homologue (Kajfasz *et al.*, 2010). *In vitro* transcription experiments further showed that the effect of SpxA1 or SpxA2 on gene expression is by direct action on RNA polymerase, as shown with the *ahpC* promoter (Kajfasz *et al.*, 2015). The *spxA1* gene is located near *ribF*, *truB*, and *pst* operon; further, *spxA1* appears to be co-transcribed and translationally coupled with a putative gene similar to Bs *yktA*. *spxA2* is located near genes involved in DNA repair such as *recA*, *ruvA*, *mutS*, and *mutL*. These genetic contexts appear to be similar in related species such as *S. pyogenes* and *S. sanguinis*.

In *S. mutans* the ClpXP protease is responsible for SpxA1 and SpxA2 degradation. The evidence comes from genetic experiments in which the pleiotropic phenotype of a *ΔclpP* strain was partially reversed by deletion of either *spxA1* or *spxA2* (Kajfasz *et al.*, 2009). In a *ΔclpX* mutant, which display similar growth defects as *ΔclpP*, interestingly, only deletion of *spxA1* reversed the pleiotropic phenotype. This observation thus appears to suggest that SpxA2 might be subjected to degradation in a ClpX-independent fashion. Consistent with the role of ClpXP, cells lacking either *clpX* or *clpP*

display elevated Spx levels (Kajfasz *et al.*, 2009). Activation of Spx-controlled genes in response to hydrogen peroxide only occurs in cells harboring SpxA1, which implies that SpxA2 is not active under those conditions (Kajfasz *et al.*, 2017). Further evidence for a distinct role for SpxA2 also emerged from *in vitro* transcription experiments in which SpxA1, but not SpxA2, is able to efficiently induce the expression of *sodA* and *tpx* (Kajfasz *et al.*, 2015). This appears to suggest that specific determinants within the Spx structure allows for the selective activation of DNA promoters.

Cells lacking *spxA1*, *spxA2*, or *spxA1* and *spxA2* display distinct phenotypes (Kajfasz *et al.*, 2010). Unlike the *ΔspxA2* strain, *ΔspxA1* cells present a growth defect under aerobic and anaerobic conditions. Interestingly, this effect is further increased in a double *ΔspxA1 ΔspxA2* mutant. Additionally, *S. mutans* cells lacking SpxA2 are unable to form cell chains, while *ΔspxA1* cells tend to form longer chains than WT; the double mutant displays a phenotype that resembles the wild-type strain. Inactivation of *spxA1* also reduced long-term survival at low pH, tolerance to diamide and hydrogen peroxide, and ability to colonize the teeth of rats, while deletion of *spxA2* had a minor effect (Galvão et al., 2017; Kajfasz et al., 2010). Importantly, inactivation of both *spx* genes results in a more dramatic effect compared to *ΔspxA1*, thus suggesting that *spxA2* plays a secondary role. Consistent with the observation that *ΔspxA1 ΔspxA2 > ΔspxA1 > ΔspxA2* were highly sensitive to diamide and hydrogen peroxide, microarray data of exponential-phase and hydrogen peroxide-treated cells showed that SpxA1 is the driver of the expression of genes involved in the oxidative stress response. Genes encoding the thioredoxin reductase (*trxB*), superoxide dismutase (*sod*), alkyl hydroperoxidase reductases (*ahpC* and *ahpF*), and glutathione reductase (*gor*), for example, were shown to be primarily under SpxA1 control (Kajfasz et al., 2010; 2017). Although those genes were also downregulated in a *ΔspxA2* mutant (Kajfasz *et al.*, 2010), most of them were not induced in response to hydrogen peroxide (Kajfasz *et al.*, 2017). Instead, SpxA2 seems to be important for the expression of genes involved in cell division, cell envelope, and fatty acid metabolism (Kajfasz *et al.*, 2010), suggesting that other yet-

to-be-determined conditions likely lead to the specific activation of the SpxA2 regulon. These results are therefore consistent with the hypothesis of specialization of the different Spx homologues.

1.10 Perspectives

The Spx protein was first described and studied in *Bacillus subtilis*. Later on, the importance of Spx proteins was elucidated in other bacterial species including human and animal pathogens, strains with biotechnological applications, and some members of the gut microbiome. The role of Spx-like proteins seems to be more diverse than previously anticipated, and Spx proteins have been linked to oxidative stress, cell wall stress, virulence, and biofilm formation, for instance. Furthermore, some bacterial species contain more than one Spx paralog, which suggests that additional environmental conditions lead to and require the activation of this set of proteins. The conditions that result in the activation of these specific Spx homologues, therefore, needs to be explored in detail to elucidate their biological role, and further studies are also needed to reveal the molecular mechanisms involved in their activation. *Bacillus subtilis*, for example, provides a unique example of how a single Spx protein can be activated under unrelated conditions employing orthogonal molecular strategies. And also, it provides an example of how two Spx paralogs can interact with distinct holoforms of the RNA polymerase. Some of the key features of Spx proteins such as the redox-sensing switch and the RPI motif, which were thought to be essential, appear to be dispensable in some Spx paralogs. These facts, hence, demand a redefinition of the characteristic features that distinguishes Spx-like proteins. For instance, some Spx paralogs do not contain either the CXXC or RPI motifs, yet are competent to modulate gene expression. While other Spx proteins possess both motifs, however, they can control their regulon without changes in the oxidation state of its redox-sensing switch. How the oxidation state of Spx proteins affects the set of controlled genes must also be addressed. Some evidence additionally appears to indicate that Spx might be subjected to other post-translational modifications,

which might also affect its function and the set of genes that are under their control. All this diversity in terms of number of paralogues, mechanisms of activation, their possible interaction with different RNA polymerase holoforms, and the range of possible post-translational modifications implicate that a vast diversity of conditions might result in activation of Spx proteins. Those conditions and the biological relevance of the activation of Spx must be the subject of future research.

1.11 References

Antelmann, H., Scharf, C., Hecker, M., and Hecker, M. (2000). Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis. *J Ba*cteriol *182*, 4478–4490.

Battesti, A., and Gottesman, S. (2013). Roles of adaptor proteins in regulation of bacterial proteolysis. *Curr Opin Microbiol 16*, 140–147.

Borezee, E., Msadek, T., Durant, L., and Berche, P. (2000). Identification in *Listeria monocytogenes* of MecA, a homologue of the *Bacillus subtilis* competence regulatory protein. *J Bacteriol 182*, 5931–5934.

Chan, C.M., Garg, S., Lin, A.A., and Zuber, P. (2012). *Geobacillus thermodenitrificans* YjbH recognizes the C-terminal end of *Bacillus subtilis* Spx to accelerate Spx proteolysis by ClpXP. *Microbiology* (Reading, Engl.) *158*, 1268–1278.

Chan, C.M., Hahn, E., and Zuber, P. (2014). Adaptor bypass mutations of *Bacillus subtilis spx* suggest a mechanism for YjbH-enhanced proteolysis of the regulator Spx by ClpXP. *Mol Microbiol 93*, 426–438.

Chatterjee, S.S., Hossain, H., Otten, S., Kuenne, C., Kuchmina, K., Machata, S., Domann, E., Chakraborty, T., and Hain, T. (2006). Intracellular gene expression profile of *Listeria monocytogenes*. *Infect Immun 74*, 1323–1338.

Chi, B.K., Kobayashi, K., Albrecht, D., Hecker, M., and Antelmann, H. (2010). The paralogous MarR/DUF24-family repressors YodB and CatR control expression of the catechol dioxygenase CatE in *Bacillus subtilis*. *J Bacteriol 192*, 4571–4581.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O.P., Devine, K.M., and Dijl, J.M.V. (2002). A Novel Class of Heat and Secretion Stress-Responsive Genes Is Controlled by the Autoregulated CssRS Two-Component System of *Bacillus subtilis*. *J Bacteriol 184*, 5661–5671.

Duwat, P., Ehrlich, S.D., and Gruss, A. (1999). Effects of metabolic flux on stress response pathways in *Lactococcus lactis*. *Mol Microbiol 31*, 845–858.

Eiamphungporn, W., and Helmann, J.D. (2008). The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. *Mol Microbiol 67*, 830–848.

Elsholz, A.K.W., Hempel, K., Michalik, S., Gronau, K., Becher, D., Hecker, M., and Gerth, U. (2011a). Activity Control of the ClpC Adaptor McsB in *Bacillus subtilis*. *J Bacteriol 193*, 3887–3893.

Elsholz, A.K.W., Turgay, K., Michalik, S., Hessling, B., Gronau, K., Oertel, D., Mader, U., Bernhardt, J., Becher, D., Hecker, M., et al. (2012). Global impact of protein arginine phosphorylation on the physiology of *Bacillus subtilis*. *Proc Natl Acad Sci USA 109*, 7451–7456.

Elsholz, A.K.W., Hempel, K., Pöther, D.-C., Becher, D., Hecker, M., and Gerth, U. (2011b). CtsR inactivation during thiol-specific stress in low GC, Gram+ bacteria. *Mol Microbiol 79*, 772–785.

Engman, J., and Wachenfeldt, von, C. (2015). Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. *Mol Microbiol 95*, 51–63.

Engman, J., Rogstam, A., Frees, D., Ingmer, H., and Wachenfeldt, von, C. (2012). The YjbH adaptor protein enhances proteolysis of the transcriptional regulator Spx in *Staphylococcus aureus*. *J Bacteriol 194*, 1186–1194.

Faulkner, M.J., and Helmann, J.D. (2011). Peroxide stress elicits adaptive changes in bacterial metal ion homeostasis. *Antioxid Redox Signal 15*, 175–189.

Frees, D., Varmanen, P., and Ingmer, H. (2001). Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in *Lactococcus lactis*. *Mol Microbiol 41*, 93–103.

Fuangthong, M., Herbig, A.F., Bsat, N., and Helmann, J.D. (2002). Regulation of the *Bacillus subtilis fur* and *perR* genes by PerR: not all members of the PerR regulon are peroxide inducible. *J Bacteriol 184*, 3276–3286.

Fuhrmann, J., Schmidt, A., Spiess, S., Lehner, A., Turgay, K., Mechtler, K., Charpentier, E., and Clausen, T. (2009). McsB is a protein arginine kinase that phosphorylates and inhibits the heat-shock regulator CtsR. *Science* (New York, N.Y.) *324*, 1323–1327.

Gaballa, A., and Helmann, J.D. (2002). A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in *Bacillus subtilis*. *Mol Microbiol 45*, 997–1005.

Gaballa, A., Antelmann, H., Hamilton, C.J., and Helmann, J.D. (2013). Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx. *Microbiology 159*, 2025–2035.

Galvão, L.C.C., Rosalen, P.L., Rivera-Ramos, I., Franco, G.C.N., Kajfasz, J.K., Abranches, J., Bueno-Silva, B., Koo, H., and Lemos, J.A. (2017). Inactivation of the *spxA1* or *spxA2* gene of *Streptococcus mutans* decreases virulence in the rat caries model. *Mol Oral Microbiol 32*, 142–153.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009). The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *J Bacteriol 191*, 1268–1277.

Gohring, N., Fedtke, I., Xia, G., Jorge, A.M., Pinho, M.G., Bertsche, U., and Peschel, A. (2011). New role of the disulfide stress effector YjbH in β-lactam susceptibility of *Staphylococcus aureus*. *Antimicrob Agents Chemother 55*, 5452–5458.

Hayashi, K., Ohsawa, T., Kobayashi, K., Ogasawara, N., and Ogura, M. (2005). The H_2O_2 stress-responsive regulator PerR positively regulates *srfA* expression in *Bacillus subtilis*. J Bacteriol *187*, 6659–6667.

Helmann, J.D. (2016). *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Curr Opin Microbiol 30*, 122–132.

Herbig, A.F., and Helmann, J.D. (2001). Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol Microbiol 41*, 849–859.

Huang, X., Gaballa, A., Cao, M., and Helmann, J.D. (1999). Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, sigma W. *Mol Microbiol 31*, 361–371.

Hyyryläinen, H.L., Bolhuis, A., Darmon, E., Muukkonen, L., Koski, P., Vitikainen, M., Sarvas, M., Prágai, Z., Bron, S., van Dijl, J.M., et al. (2001). A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol Microbiol 41*, 1159–1172.

Jervis, A.J., Thackray, P.D., Houston, C.W., Horsburgh, M.J., and Moir, A. (2007). SigM-Responsive Genes of *Bacillus subtilis* and Their Promoters. *J Bacteriol 189*, 4534–4538.

Jousselin, A., Kelley, W.L., Barras, C., Lew, D.P., and Renzoni, A. (2013). The *Staphylococcus aureus* thiol/oxidative stress global regulator Spx controls *trfA*, a gene implicated in cell wall antibiotic resistance. *Antimicrob Agents Chemother 57*, 3283–3292.

Kajfasz, J.K., Ganguly, T., Hardin, E.L., Abranches, J., and Lemos, J.A. (2017). Transcriptome responses of *Streptococcus mutans* to peroxide stress: identification of novel antioxidant pathways regulated by Spx. *Sci Rep 7*, 16018.

Kajfasz, J.K., Martinez, A.R., Rivera-Ramos, I., Abranches, J., Koo, H., Quivey, R.G., and Lemos, J.A. (2009). Role of Clp proteins in expression of virulence properties of *Streptococcus mutans*. *J Bacteriol 191*, 2060–2068.

Kajfasz, J.K., Mendoza, J.E., Gaca, A.O., Miller, J.H., Koselny, K.A., Giambiagi-deMarval, M., Wellington, M., Abranches, J., and Lemos, J.A. (2012). The Spx Regulator Modulates Stress Responses and Virulence in *Enterococcus faecalis*. *Infect Immun 80*, 2265–2275.

Kajfasz, J.K., Rivera-Ramos, I., Abranches, J., Martinez, A.R., Rosalen, P.L., Derr, A.M., Quivey, R.G., and Lemos, J.A. (2010). Two Spx proteins modulate stress tolerance, survival, and virulence in *Streptococcus mutans*. *J Bacteriol 192*, 2546–2556.

Kajfasz, J.K., Rivera-Ramos, I., Scott-Anne, K., Gregoire, S., Abranches, J., and Lemos, J.A. (2015). Transcription of Oxidative Stress Genes Is Directly Activated by SpxA1 and, to a Lesser Extent, by SpxA2 in *Streptococcus mutans*. *J Bacteriol 197*, 2160–2170.

Kawai, Y., Mercier, R., Wu, L.J., Domínguez-Cuevas, P., Oshima, T., and Errington, J. (2015). Cell growth of wall-free L-form bacteria is limited by oxidative damage. *Curr Biol 25*, 1613–1618.

Kirstein, J., Dougan, D.A., Gerth, U., Hecker, M., and Turgay, K. (2007). The tyrosine kinase McsB is a regulated adaptor protein for ClpCP. *Embo* J *26*, 2061–2070.

Kirstein, J., MoliEre, N., Dougan, D.A., and Turgay, K. (2009). Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat. Rev. Microbiol. 7*, 589–599.

Kommineni, S., Garg, S.K., Chan, C.M., and Zuber, P. (2011). YjbH-enhanced proteolysis of Spx by ClpXP in *Bacillus subtilis* is inhibited by the small protein YirB (YuzO). *J Bacteriol 193*, 2133–2140.

Larsson, J.T., Rogstam, A., and Wachenfeldt, von, C. (2007). YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. *Mol Microbiol 66*, 669–684.

Lee, J.-W., and Helmann, J.D. (2006). The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. *Nature 440*, 363–367.

Lee, J.-W., Soonsanga, S., and Helmann, J.D. (2007). A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc Natl Acad Sci USA 104*, 8743–8748.

Leelakriangsak, M., and Zuber, P. (2007). Transcription from the P_3 promoter of the *Bacillus* subtilis spx gene is induced in response to disulfide stress. *J Bacteriol 189*, 1727–1735.

Leelakriangsak, M., Huyen, N.T.T., Töwe, S., van Duy, N., Becher, D., Hecker, M., Antelmann, H., and Zuber, P. (2008). Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis. Mol Microbiol 67*, 1108–1124.

Leelakriangsak, M., Kobayashi, K., and Zuber, P. (2007). Dual negative control of *spx* transcription initiation from the P_3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *J Bacteriol 189*, 1736–1744.

Li, S., Rosen, B.P., Borges-Walmsley, M.I., and Walmsley, A.R. (2002). Evidence for Cooperativity between the Four Binding Sites of Dimeric ArsD, an As(III)-responsive Transcriptional Regulator. *J Biol Chem 277*, 25992–26002.

Marciniak, B.C., Trip, H., van-der Veek, P.J., and Kuipers, O.P. (2012). Comparative transcriptional analysis of *Bacillus subtilis* cells overproducing either secreted proteins, lipoproteins or membrane proteins. *Microb. Cell Fact. 11*, 66.

Myers, J.T., Tsang, A.W., and Swanson, J.A. (2003). Localized Reactive Oxygen and Nitrogen Intermediates Inhibit Escape of *Listeria monocytogenes* from Vacuoles in Activated Macrophages. *J Immunol 171*, 5447–5453.

Nakano, M.M., Hajarizadeh, F., Zhu, Y., and Zuber, P. (2001). Loss-of-function mutations in *yjbD* result in ClpX- and ClpP-independent competence development of *Bacillus subtilis. Mol Microbiol 42*, 383–394.

Nakano, M.M., Kominos-Marvell, W., Sane, B., Nader, Y.M., Barendt, S.M., Jones, M.B., and Zuber, P. (2014). *spxA2*, encoding a regulator of stress resistance in *Bacillus anthracis*, is controlled by SaiR, a new member of the Rrf2 protein family. *Mol Microbiol 94*, 815–827.

Nakano, M.M., Lin, A., Zuber, C.S., Newberry, K.J., Brennan, R.G., and Zuber, P. (2010). Promoter recognition by a complex of Spx and the C-terminal domain of the RNA polymerase alpha subunit. *PLoS One 5*, e8664.

Nakano, S., Erwin, K.N., Ralle, M., and Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol 55*, 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A.D., and Zuber, P. (2003a). Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proc Natl Acad Sci USA 100*, 13603–13608.

Nakano, S., Nakano, M.M., Zhang, Y., Leelakriangsak, M., and Zuber, P. (2003b). A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proc Natl Acad Sci USA 100*, 4233–4238.

Nakano, S., Zheng, G., Nakano, M.M., and Zuber, P. (2002). Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis. J Bacteriol 184*, 3664–3670.

Newberry, K.J., Nakano, S., Zuber, P., and Brennan, R.G. (2005). Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. *Proc Natl Acad Sci USA 102*, 15839–15844.

Nicolas, P., Nicolas, P., Mader, U., Mäder, U., Dervyn, E., Dervyn, E., Rochat, T., Rochat, T., Leduc, A., Leduc, A., et al. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* (New York, N.Y.) *335*, 1103–1106.

Pamp, S.J., Frees, D., Engelmann, S., Hecker, M., and Ingmer, H. (2006). Spx Is a Global Effector Impacting Stress Tolerance and Biofilm Formation in *Staphylococcus aureus*. *J Bacteriol 188*, 4861–4870.

Reder, A., Höper, D., Weinberg, C., Gerth, U., Fraunholz, M., and Hecker, M. (2008). The Spx paralogue MgsR (YqgZ) controls a subregulon within the general stress response of *Bacillus subtilis*. *Mol Microbiol 69*, 1104–1120.

Reder, A., Pöther, D.-C., Gerth, U., and Hecker, M. (2012). The modulator of the general stress response, MgsR, of *Bacillus subtilis* is subject to multiple and complex control mechanisms. *Environ. Microbiol. 14*, 2838–2850.

Reniere, M.L., Whiteley, A.T., and Portnoy, D.A. (2016). An *In Vivo* Selection Identifies *Listeria monocytogenes* Genes Required to Sense the Intracellular Environment and Activate Virulence Factor Expression. *PLoS Pathog 12*, e1005741–27.

Renzoni, A., Andrey, D.O., Jousselin, A., Barras, C., Monod, A., Vaudaux, P., Lew, D., and Kelley, W.L. (2011). Whole genome sequencing and complete genetic analysis reveals novel pathways to glycopeptide resistance in *Staphylococcus aureus*. *PLoS One 6*, e21577.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinova, A., Korelusova, J., Leduc, A., Bessieres, P., Dervyn, E., Krasny, L., and Noirot, P. (2012). Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis. Nucleic Acids Res 40*, 9571–9583.

Rogstam, A., Larsson, J.T., Kjelgaard, P., and Wachenfeldt, von, C. (2007). Mechanisms of Adaptation to Nitrosative Stress in *Bacillus subtilis*. *J Bacteriol 189*, 3063–3071.

Rojas-Tapias, D.F., and Helmann, J.D. (2018a). Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis. Mol Microbiol 107*, 659–674.

Rojas-Tapias, D.F., and Helmann, J.D. (2018b). Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. PLoS Genet *14*, e1007531.

Runde, S., MoliEre, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., Gerth, U., Hecker, M., and Turgay, K. (2014). The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *Bacillus subtilis*. *Mol Microbiol 91*, 1036–1052.

Salazar, M.E., and Laub, M.T. (2015). Temporal and evolutionary dynamics of two-component signaling pathways. *Curr Opin Microbiol 24*, 7–14.

Thackray, P.D., and Moir, A. (2003). SigM, an Extracytoplasmic Function Sigma Factor of *Bacillus subtilis*, Is Activated in Response to Cell Wall Antibiotics, Ethanol, Heat, Acid, and Superoxide Stress. *J Bacteriol 185*, 3491–3498.

Trentini, D.B., Suskiewicz, M.J., Heuck, A., Kurzbauer, R., Deszcz, L., Mechtler, K., and Clausen, T. (2016). Arginine phosphorylation marks proteins for degradation by a Clp protease. Nature *539*, 48–53.

Turner, M.S., Tan, Y.P., and Giffard, P.M. (2007). Inactivation of an iron transporter in *Lactococcus lactis* results in resistance to tellurite and oxidative stress. Appl Environ Microbiol *73*, 6144–6149.

Veiga, P., Bulbarela-Sampieri, C., Furlan, S., Maisons, A., Chapot-Chartier, M.-P., Erkelenz, M., Mervelet, P., Noirot, P., Frees, D., Kuipers, O.P., et al. (2007). SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. J Biol Chem *282*, 19342–19354.

Wang, C., Fan, J., Niu, C., Wang, C., Villaruz, A.E., Otto, M., and Gao, Q. (2010). Role of *spx* in biofilm formation of *Staphylococcus epidermidis*. FEMS *Immunol Med Microbiol 59*, 152–160.

Wecke, T., Bauer, T., Harth, H., Mäder, U., and Mascher, T. (2011). The rhamnolipid stress response of *Bacillus subtilis*. *FEMS Microbiol Lett 323*, 113–123.

Whiteley, A.T., Ruhland, B.R., Edrozo, M.B., and Reniere, M.L. (2017). A Redox-Responsive Transcription Factor Is Critical for Pathogenesis and Aerobic Growth of *Listeria monocytogenes. Infect Immun 85*, 395–12.

Wu, J., and Rosen, B.P. (1993). The *arsD* gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon. *Mol Microbiol 8*, 615–623.

Wurtzel, O., Sesto, N., Mellin, J.R., Karunker, I., Edelheit, S., Bécavin, C., Archambaud, C., Cossart, P., and Sorek, R. (2012). Comparative transcriptomics of pathogenic and non-pathogenic *Listeria* species. *Mol Syst Biol 8*, 583.

Zhang, Y., and Zuber, P. (2007). Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. *J Bacteriol 189*, 7669–7680.

Zuber, P. (2004). Spx-RNA Polymerase Interaction and Global Transcriptional Control during Oxidative Stress. *J Bacteriol 186*, 1911–1918.

CHAPTER II: Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*

The present Chapter is a modified version of the already published manuscript:

Rojas-Tapias, D.F., and Helmann, J.D. (2018a). Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in Bacillus subtilis. Mol Microbiol *107*, 659–674.

2.1 Summary

The transcription factor Spx is the master regulator of the disulfide stress response in *Bacillus subtilis*. Intriguingly, the activation of Spx by diamide relies entirely on posttranslational regulatory events in spite of the complex transcriptional control of the *spx* gene. Here, we show that cell wall stress, but not membrane stress, also results in induction of the Spx regulon. Remarkably, two major differences were found regarding the mechanism of induction of Spx under cell wall stress in comparison to disulfide stress. First, transcriptional induction of the *spx* gene from a σ^M-dependent promoter is required for accumulation of Spx in response to cell wall stress. Second, activation of the Spx regulon during cell wall stress is not accompanied by oxidation of the Spx disulfide switch. Finally, we demonstrate that cells lacking Spx have increased sensitivity towards antibiotics inhibiting both early and late steps in peptidoglycan synthesis, suggesting that the Spx regulon plays an important adaptive role in the cell wall stress response. This study expands the functional role of the Spx regulon and reveals novel regulatory mechanisms that result in induction of Spx in *B. subtilis*.

2.2 Introduction

Spx is a pleiotropic transcription factor that controls the disulfide-stress response in *Bacillus subtilis* (Nakano *et al.*, 2003), and other low-GC Gram-positive bacteria including major pathogens (Pamp *et al.*, 2006; Veiga *et al.*, 2007; Kajfasz *et al.*, 2010; Barendt *et al.*, 2013). An *spx* null mutant is sensitive to diamide, an electrophilic agent that selectively leads to the formation of intramolecular and intermolecular disulfide bonds (Nakano *et al.*, 2003). Spx belongs to the ArsC family of transcription factors, and controls gene expression by direct binding to the C-terminal domain of the alpha subunit of the RNA polymerase (Newberry *et al.*, 2005). Activation of Spx results in the specific induction or repression of more than 140 transcriptional units, including genes involved in the synthesis of bacillithiol and cysteine, redox homeostasis, and proteolytic control (Rochat *et al.*, 2012).

The production of Spx is tightly regulated at several levels. At the transcriptional level, *spx* expression is affected by four promoters responsive to different sigma factors: σ^A, $\sigma^{M/X/W}$, σ^M, and σ^B (Antelmann *et al.*, 2000; Leelakriangsak and Zuber, 2007; Jervis *et al.*, 2007; Luo and Helmann, 2012). Two repressors, PerR and YodB, also control the expression of *spx* (Leelakriangsak *et al.*, 2007). At the post-translational level, the cytoplasmic concentration of Spx is tightly controlled by proteolysis. Under non-stress conditions, the Spx protein is translated but actively unfolded and degraded via the ATP-dependent protease ClpXP in a process dependent on the adaptor protein YjbH (Nakano *et al.*, 2002; Larsson *et al.*, 2007; Zhang and Zuber, 2007; Garg *et al.*, 2009). Conversely, upon disulfide treatment the proteolytic activity of ClpXP is reduced and YjbH aggregates, allowing for Spx accumulation (Zhang and Zuber, 2007; Garg *et al.*, 2009; Engman and von Wachenfeldt, 2015). The ability of Spx to regulate gene expression is also influenced by the oxidation state of its redox-sensing switch. The N-terminal domain of Spx contains a CXXC motif that becomes oxidized under conditions that increase disulfide bond formation (disulfide stress). *In vitro* studies have shown that the formation of this disulfide bond in Spx provokes a conformational change in the RNA polymerase-Spx-DNA

promoter ternary complex that stimulates the expression of *trxA* and *trxB* (Nakano *et al.*, 2005). Recent evidence, however, suggests that oxidation of the redox-sensing switch is not essential for induction of all Spx-controlled genes (Rochat *et al.*, 2012).

Bacteria respond to cell envelope damage through multiple regulatory systems (Jordan *et al.*, 2008; Helmann, 2016), often involving extracytoplasmic (ECF) σ factors (σ^{ECF}). The specific molecular signals to which ECF σ factors respond remain largely unknown. One notable exception is σ^{V}, which responds strongly and specifically to lysozyme (Guariglia-Oropeza and Helmann, 2011; Ho *et al.*, 2011), and this response involves a direct protein interaction between lysozyme and the anti-σ factor (Hastie *et al.*, 2016). Although the precise molecular signals remain unknown, σ^{M}, σ^{W} and σ^{X} respond to inhibitors of peptidoglycan (PG) synthesis, membrane-active compounds, and antimicrobial cationic peptides, respectively (Helmann, 2016). Interestingly, the *yjbC-spx* operon contains two promoters that appear to be regulated by ECF σ factors (Jervis *et al.*, 2007; Eiamphungporn and Helmann, 2008), and cell envelope antibiotics induce *spx* and Spx-dependent genes (Cao *et al.*, 2002b; Eiamphungporn and Helmann, 2008; Wecke *et al.*, 2011; Kawai *et al.*, 2015).

Studies employing diamide to generate disulfide stress have provided the foundation for our understanding of the Spx regulon. In contrast, activation of the Spx regulon in response to other signals is poorly understood. Here, we show that Spx is required for a robust cell wall stress response and, unlike disulfide stress, transcriptional induction of *spx* from a σ^{M}-regulated promoter is required to ensure full induction of the Spx regulon. We also show that oxidation of the Spx redox-sensing switch under cell wall stress plays a more limited role compared to diamide stress in induction of Spx-controlled genes, and that induction of the Spx regulon confers protection against antibiotics.

2.3 Results

2.3.1 Cell wall stress leads to upregulation of *spx*

The *spx* gene is located in a bicistronic operon with *yjbC*, which encodes a putative acetyltransferase. Several promoters appear to be responsible for the transcription of *spx*, including one σ^A promoter (P_A) located in the intergenic region between *yjbC* and *spx* (Leelakriangsak and Zuber, 2007), a σ^B-dependent promoter (P_B) located upstream of *yjbC* (Antelmann *et al.*, 2000), and two promoters controlled by σ^{ECF} factors (Cao *et al.*, 2002a; Jervis *et al.*, 2007). One putative σ^{ECF}-dependent promoter is upstream of *yjbC* (P_{M1}) and another is in the intergenic region between *yjbC* and *spx* (P_{M2}) (Fig 2.1A). Additionally, two repressors (i.e. PerR and YodB) bind the intergenic region of *spx* and *yjbC* (Leelakriangsak *et al.*, 2007). This complex transcriptional context suggests that induction of the *spx* gene in response to stress likely contributes to activation of the Spx regulon.

Both P_{M1} and P_{M2} were detected by 5'-RACE in cells induced for σ^M expression (Jervis *et al.*, 2007), but the contribution of these promoters to *spx* expression in response to stress has not been defined. Since σ^M responds to cell wall antibiotics, we first studied the transcriptional profile of *spx* in cells treated with various cell wall-active antibiotics. Two transcripts were identified in an RNA (northern) blot with molecular sizes that corresponded to promoters located in the upstream region of *yjbC* and the intergenic region of the operon: ~1.3 kb and ~0.5 kb, respectively (Fig 2.1B). There was no significant change in the level of the *yjbC-spx* mRNA after 10 min. or 40 min. of diamide treatment. The monocistronic *spx* transcript was somewhat elevated after 40 min. compared to the 10 min. sample in both the diamide treated and the control cells. In contrast, treatment with PG synthesis inhibitors resulted in a dramatic increase in the expression of the *yjbC-spx* mRNA, which was most apparent at the 40 min. timepoint. These results suggest that P_B or P_{M1} likely account for transcriptional induction of *spx* under cell wall stress.

To further evaluate the contribution of the intergenic promoters to the cell wall stress response, we constructed a transcriptional fusion of the region encompassing the P_A and P_{M2} promoters to the *lacZ* gene (i.e. $P_{M2,A}$-*lacZ*) (Fig 2.1C) and monitored induction using northern blots. Compared to the untreated control, both fosfomycin and vancomycin resulted in a decrease in the activity of the intergenic promoters, suggesting that the smaller RNA transcript previously observed after treatment with PG inhibitors (Fig 2.1B) may arise from post-transcriptional processing. In the presence of diamide, a modest increase was observed after 10 min. (Fig 2.1C). We next assessed if the observed regulation occurred at P_A, which is regulated by YodB and PerR. As observed using the $P_{M2,A}$-*lacZ* fusion, cell wall stress resulted in repression of P_A-*lacZ* compared to the untreated control, while a marked induction of P_A was observed in presence of diamide (Fig 2.1D), as previously reported (Leelakriangsak *et al.,* 2007). Because the antibiotics tested inhibited different steps in the biosynthesis of peptidoglycan, we conclude that cell wall stress, rather than recognition of any one antibiotic, accounts for the induction of *spx* and this induction originates from promoter(s) upstream of *yjbC*.

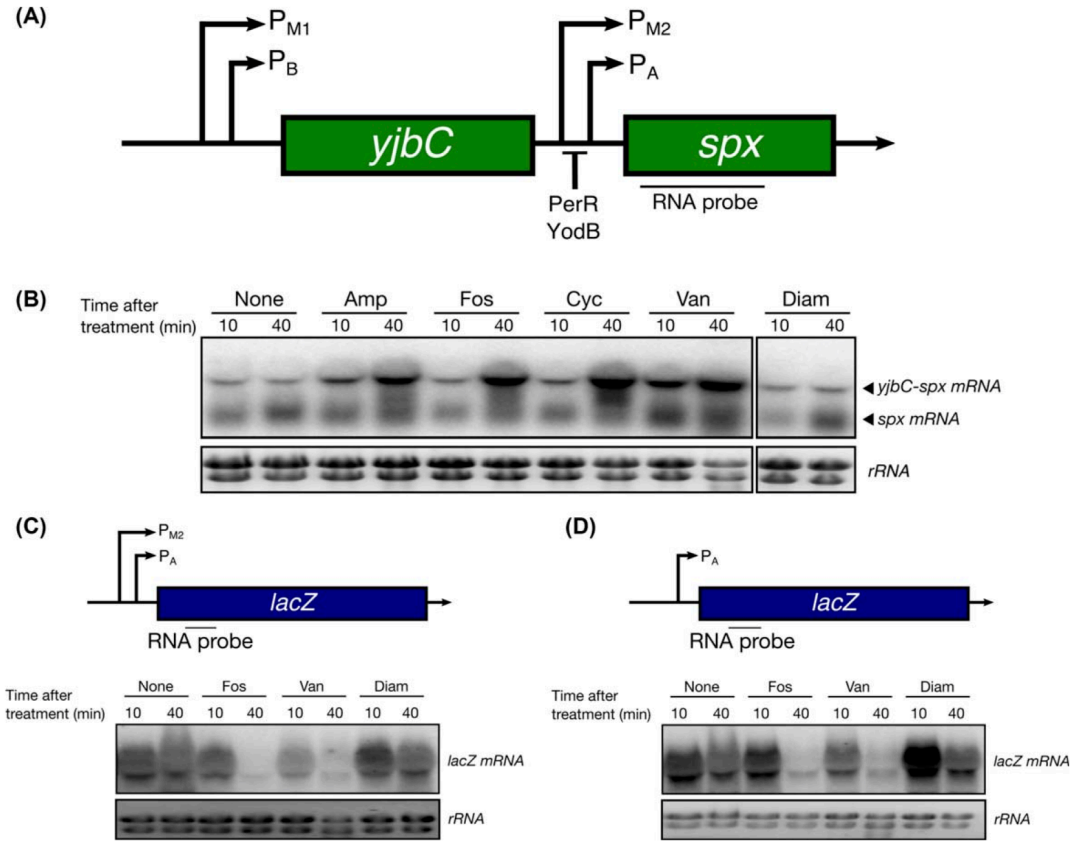

FIG 2.1 THE SPX GENE IS INDUCED IN RESPONSE TO CELL WALL STRESS.

A) Organization of the *yjbC-spx* operon and location of the *spx* RNA probe. B) Northern blot analysis using an *spx* RNA probe and 5 mg of total RNA per lane. Samples were taken 10 min and 40 min after treatment with 2 mg ml^{-1} ampicillin, 200 mg ml^{-1} fosfomycin, 250 mg ml^{-1} D-cycloserine, 1 mg ml^{-1} vancomycin, and 0.5 mM diamide (standard concentrations corresponding to 2x MIC, unless otherwise stated). C) Analysis of the small transcript using a *lacZ* fusion. The intergenic promoters (i.e., P_{M2} and P_A) were fused to the *lacZ* gene, and their induction in response to fosfomycin, vancomycin and diamide was studied by northern blot. Untreated cells were used as control. The position of the *lacZ* RNA probe is depicted. D) The contribution of the P_A promoter to induction of the *spx* gene was studied as described in Fig 2.1C, with the exception that only the region encompassing the P_A promoter was chosen for the transcriptional fusion. Each blot is representative of at least two biological replicates.

2.3.2 The P_{M1} promoter drives the expression of *spx* under cell wall stress

To dissect the contribution of the various promoters upstream of *spx* to induction in response to cell wall stress, we used a GFP reporter fusion expressed ectopically from a DNA fragment containing the entire *yjbC* gene together with upstream and downstream promoters. This fusion is significantly induced after 40 min. of antibiotic treatment, but this induction is eliminated by point mutations designed to inactivate P_{M1} (Fig 2.2A, 2.2B, 2.2D, 2.2E). In contrast, mutations in the predicted P_B or P_{M2} promoters had no effect on induction, which is consistent with the fact that Δ*sigB* cells are still able to upregulate *yjbC-spx* (Fig 2.2E) and that the intergenic promoters do not contribute to induction of *spx* (Fig 2.1C) in response to cell wall stress. The location of P_{M1} upstream of *yjbC* is in agreement with the size of the induced transcript as observed using northern blot analysis (Fig 2.1B). We conclude that induction of the *spx* gene is driven by P_{M1} in response to cell wall stress.

To determine which ECF sigma factor(s) were responsible for induction of P_{M1}, we first monitored the activation of the major ECF σ factors (i.e. σ^M, σ^W, and σ^X) in response to cell wall stress (Helmann, 2016). Since the genes encoding σ^M, σ^W, and σ^X are positively autoregulated, the measurement of their mRNA levels reflects their activity in response to different environmental conditions. Fosfomycin and ampicillin resulted in induction of *sigM* and *sigW*, whereas *sigX* was unresponsive (Fig 2.2C). This suggests that σ^M and/or σ^W are likely responsible for induction of *yjbC-spx*. In a *sigM* null mutant, the level of antibiotic induction was dramatically reduced, but not eliminated, and this phenotype was complemented by ectopic expression of σ^M (Fig 2.2D). The residual induction in the *sigM* null mutant was not due to σ^W since induction was largely unaffected in a *sigW* mutant, and a *sigW sigM* double mutant behaved similarly to the *sigM* single mutant. Further analysis revealed that σ^X also contributes to the expression of *yjbC-spx*: induction was absent in a *sigM sigX* mutant and expression was fully eliminated in the *sigM sigW sigX* triple mutant (Fig 2.2E). Together, these results indicate that σ^M is the major ECF sigma factor required for induction of *spx* in response to cell wall antibiotics.

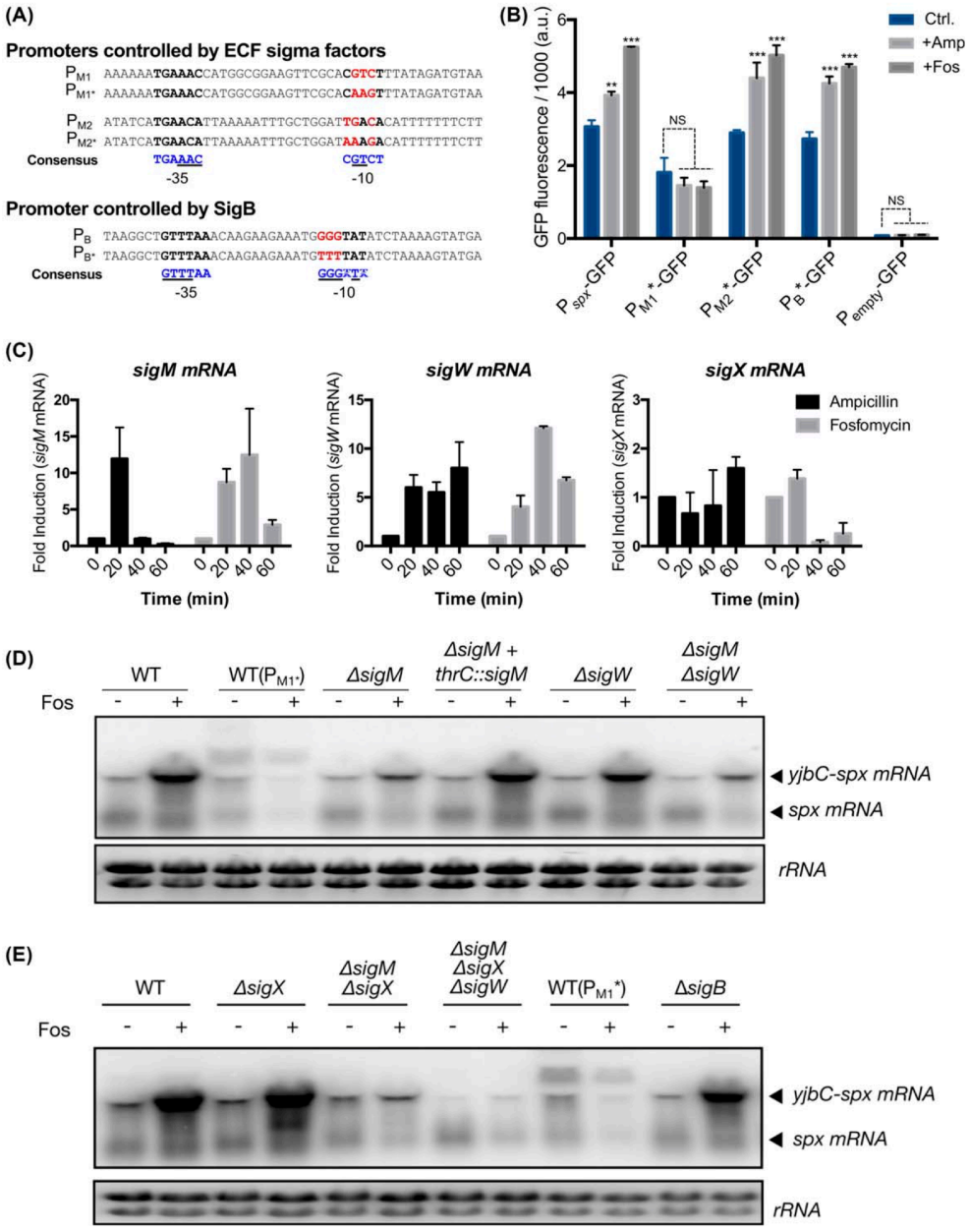

FIG 2.2 ANALYSIS OF THE SPX PROMOTER IN RESPONSE TO CELL WALL STRESS.

A) Sequences of the promoters in the *yjbC-spx* operon as stated in Fig 2.1A; additionally, the sequences of the mutant promoters are presented with an asterisk. The DNA bases in red color indicate the sites at which point mutations were introduced, and the underlined bases in the consensus sequence indicate the most conserved residues for the mentioned sigma factors. The -10 and -35 boxes are also displayed. B) The importance of the *spx* promoters was tested using transcriptional fusions of the promoters listed in Fig 2.2A with a *gfp* reporter. Cells were left untreated, or treated with ampicillin and fosfomycin, and fluorescence was measured after 47 min of treatment using flow cytometry (7 min. were allowed for maturation of GFPMut3). Error bars indicate standard error of the mean (SEM), n=3. One, two, and three asterisks indicate

significant differences with P <0.05, P <0.01, and P <0.001, respectively, as estimated using the Dunnett Test and comparing the treated samples against the control. NS indicates no significant differences. C) Induction of the ECF sigma factors under cell wall stress. The induction profile of *sigM*, *sigW*, and *sigX* was determined using RT-qPCR. Error bars indicate SEM (n=3). As observed, only the σ^M and σ^W regulons were induced under the conditions studied. D) The contribution of the ECF sigma factors for the induction of the P_{M1} promoter was tested by northern blot using an *spx* RNA probe. Samples were collected after 40 min of treatment or not with fosfomycin. A total of 5 μg of RNA was loaded per well. E) Analysis of the remaining transcript using northern blot and an *spx* RNA probe. The same conditions as in Fig 2.2D were used.

2.3.3 The Spx protein accumulates under cell wall stress

The accumulation of the Spx protein in response to disulfide stress primarily occurs through reduced proteolysis (Zuber, 2009). We therefore asked whether antibiotic stress also leads to accumulation of Spx protein, and whether induction of the P_{M1} promoter is required for this accumulation. Western blot analysis using anti-Spx antiserum reveals a dramatic increase in Spx in cells following treatment with ampicillin and fosfomycin when compared to mock-treated samples (Fig 2.3A). Several other peptidoglycan synthesis inhibitors (vancomycin, cycloserine, and cefuroxime), also led to a clear increase in Spx levels, but there was comparatively little effect after treatment with membrane active antibiotics (colistin, daptomycin, polymyxin B, and nisin) (Fig 2.3D). This pattern of induction is consistent with the role of σ^M in induction of P_{M1}, since σ^M is known to respond strongly to cell wall stress elicited by inhibitors of peptidoglycan synthesis (Eiamphungporn and Helmann, 2008; Czarny *et al.*, 2014), whereas membrane-active compounds strongly induce σ^W (Helmann, 2016). As expected, the accumulation of the Spx protein under cell wall stress was σ^M-dependent: the P_{M1}* strain was unable to accumulate Spx upon fosfomycin treatment (Fig 2.3B), even though fosfomycin is amongst the strongest inducers of Spx accumulation (Fig 2.3A). Ectopic complementation of a strain harboring the P_{M1}* promoter with an *spx* allele driven from the P_{M1} promoter (i.e. *amyE*::P_{M1}-*spx*) fully restored induction (Fig 2.3B), further validating the critical role of the P_{M1} promoter. In contrast, Spx accumulation in response to diamide was unaffected by the P_{M1*} mutation (Fig 2.3C). These results

suggest that, in contrast to disulfide stress, transcriptional activation of the *spx* gene is required for accumulation of Spx under cell wall stress.

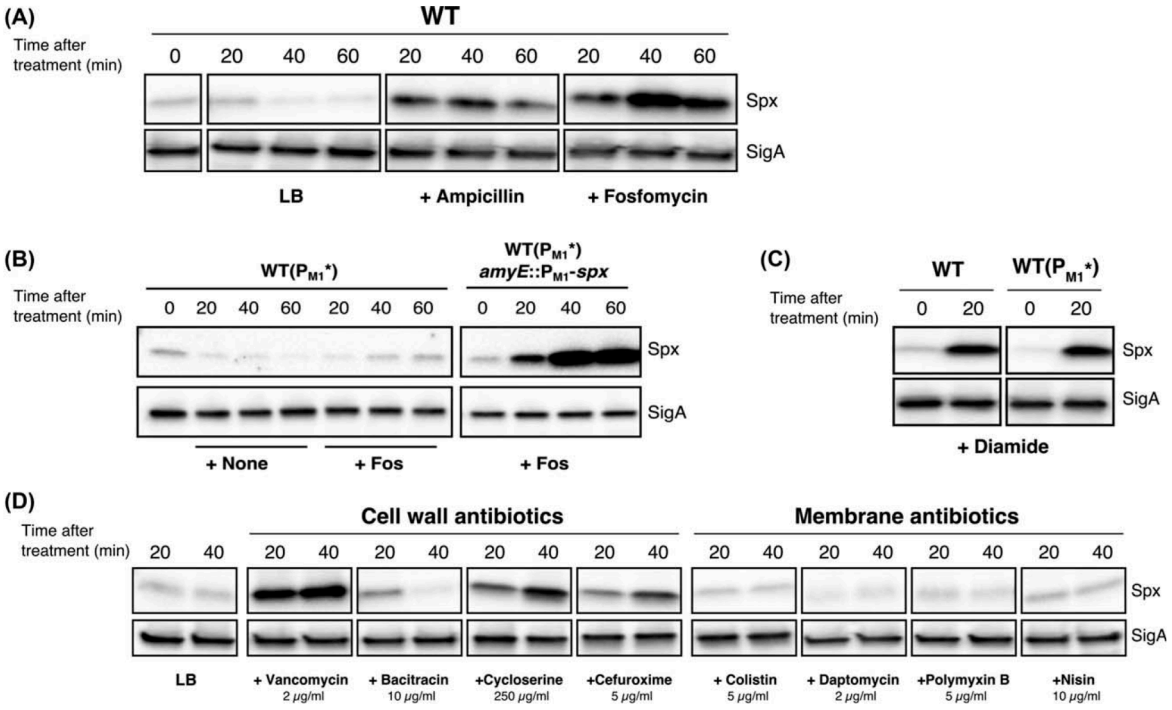

FIG 2.3. ACCUMULATION OF THE SPX PROTEIN UNDER CELL WALL STRESS REQUIRES P_{M1}.

A) The Spx protein accumulates upon treatment with ampicillin and fosfomycin as studied using western blot. B) Cells unable to induce the P_{M1} promoter were unable to accumulate Spx in the presence of fosfomycin. Complementation with an ectopic copy of *spx* driven from P_{M1} restored the wild-type phenotype. C) Accumulation of Spx in cells harboring P_{M1} or P_{M1}* under treatment with diamide display no significant differences, suggesting that induction of P_{M1} is not required for the disulfide stress response. D) PG synthesis inhibitors, but not membrane antibiotics, induce the Spx regulon as monitored using western blot. Each blot is representative of at least three biological replicates.

2.3.4 Cell wall stress activates the Spx regulon

To determine whether accumulation of Spx is correlated with induction of the Spx regulon, we monitored mRNA levels for four Spx target genes: *trxB*, *trxA*, *yjbH*, and *nfrA*, after treatment with fosfomycin (Fig 2.4A). Fosfomycin led to an Spx-dependent induction of all four genes. To investigate the dynamics of induction of the genes in the Spx regulon, we chose the *trxB* gene whose transcription fully depends on Spx. Expression of *trxB* was transient, and peaked after 20 min. and 40 min. of treatment with ampicillin and fosfomycin, respectively (Fig 2.4B). These results correlate with the interruption of late and early stages in the synthesis of PG. While ampicillin prevents PG transpeptidation, fosfomycin inhibits the first cytosolic enzyme committed in the synthesis of PG (i.e. MurAA). Cell wall damage, therefore, correlates with the induction of the Spx regulon.

Next, we studied the Spx-dependent induction of *trxB* in WT and WT(P_{M1}*) cells using a P_{trxB}-*gfp* transcriptional fusion. In the absence of antibiotic or diamide challenge, both strains displayed similar *trxB* activity. Similar induction levels were also observed in diamide-treated cells (Fig 2.4C), which was consistent with the observed Spx protein levels (Fig 2.3C), and suggests that the induction of *trxB* in response to diamide proceeds in a P_{M1}-independent fashion. In contrast, induction of *trxB* by PG synthesis inhibitors was significantly reduced, but not eliminated, in cells harboring P_{M1}* (Fig 2.4C). A similar pattern emerged when we monitored the induction dynamics of *trxB, trxA,* and *nfrA* in response to fosfomycin: induction was dramatically reduced, but not eliminated, in cells unable to activate P_{M1} (Fig 2.4D). Taken together, we conclude that cell wall stress leads to induction of the Spx regulon, transcriptional induction of *spx* from P_{M1} is required for the full induction, but post-transcriptional stabilization of Spx may also contribute to induction.

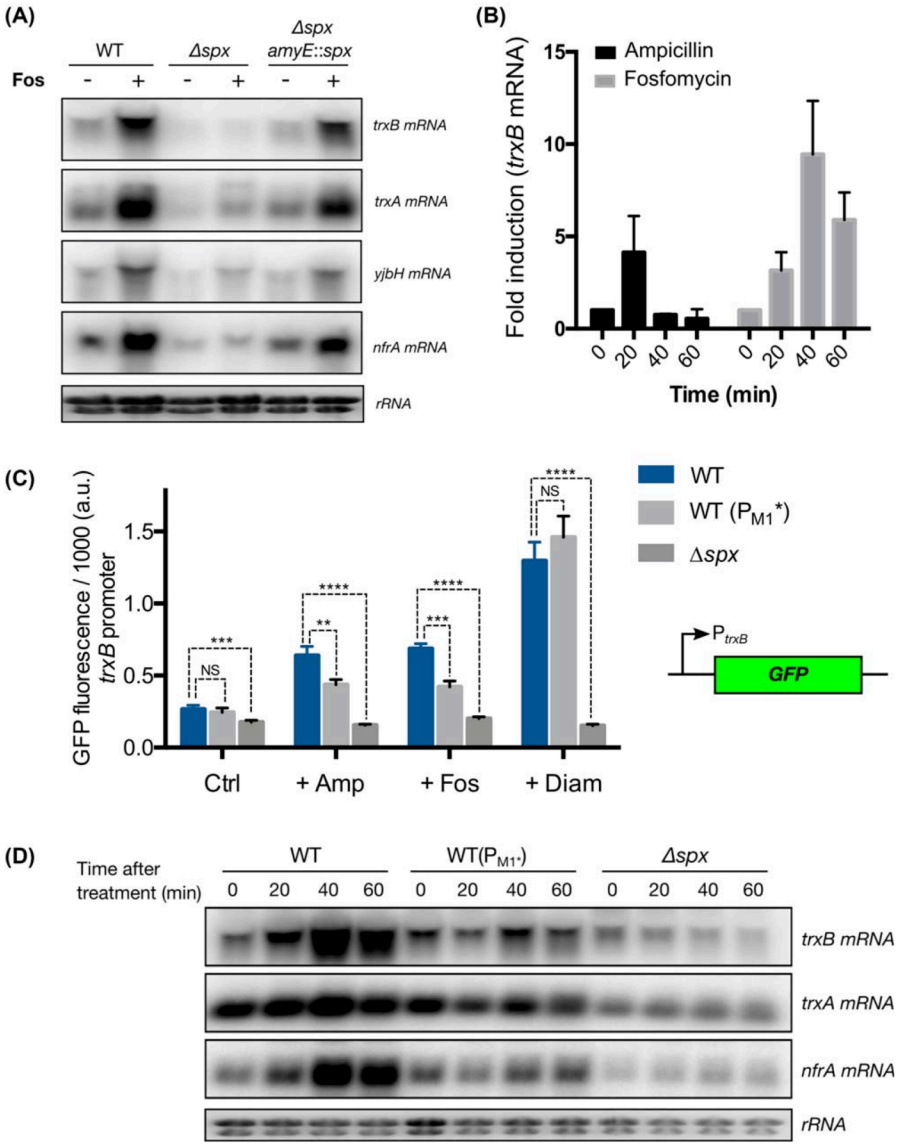

FIG 2.4 THE SPX REGULON IS INDUCED UNDER CELL WALL STRESS.

A) Northern blot analysis shows that *trxB*, *trxA*, *yjbH*, and *nfrA* are induced in response to fosfomycin treatment in an Spx-dependent fashion. The RNA was isolated after 40 min. of treatment. B) Time-course experiment, using RT-qPCR, shows the dynamics of *trxB* expression. Antibiotic concentrations were used as listed in Fig 2.1B. C) The expression of the *trxB* gene was also studied in cells unable to activate the P_{M1} promoter using a P_{trxB}-*gfp* transcriptional fusion. Fluorescence was measured by flow cytometry 47 min. after treatment or not with ampicillin, fosfomycin, and diamide. Each experiment corresponds to at least three independent experiments. Error bars indicate SEM. One, two, and three asterisks indicate significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively, as estimated using the Dunnett Test and comparing the mutant strains against WT. NS indicates no significant differences. D) Time-course experiment shows the dynamics of induction of three Spx-controlled genes in response to fosfomycin in WT, WT(P_{M1}*), and Δ*spx*.

2.3.5 The Spx protein remains primarily in its reduced state under cell wall stress

To study the redox state of Spx (Fig 2.5A) and its contribution to the induction of the regulon, we first monitored *trxB* induction using the P_{trxB}-*gfp* transcriptional fusion. Induction of *trxB* was substantially higher under disulfide stress than cell wall stress (Fig 2.5B), albeit comparable protein levels were observed in both conditions (Fig 2.3 and data not shown). Since oxidation of the Spx redox-sensing switch increases transcription of *trxB*, we therefore hypothesized that cell wall stress might be less efficient than diamide in leading to Spx oxidation (Nakano *et al.*, 2005; Rochat *et al.*, 2012).

To further study the role of Spx oxidation, we compared *trxB* induction in cells harboring *spx* or spx^{C10A} (under native control). The spx^{C10A} allele encodes a mutant Spx protein (Cys10 to Ala substitution) unable to form the canonical intramolecular disulfide bond (Fig 2.5A) and therefore is generally assumed to reflect the activity of reduced Spx protein (Nakano *et al.*, 2005). We note that Spx^{C10A} only displays a slight decrease in affinity for binding to the α subunit of RNA polymerase compared with the wild-type protein (Lin *et al.*, 2013), and that similar Spx protein levels are observed in cells harboring the wild-type or mutant Spx protein (Fig 2.5D) (Nakano *et al.*, 2005). The basal level of *trxB* expression was significantly lower in cells harboring Spx^{C10A}, even in the absence of stress, suggesting that either some Spx oxidation occurs in growing cells, or that this amino acid change impacts the ability of Spx to induce gene expression (Fig 2.5B). Diamide treatment caused a dramatic increase in *trxB* expression in wild type cells compared to cells harboring Spx^{C10A}, further validating the importance of the Spx disulfide switch in the induction of the Spx regulon under disulfide stress (Fig 2.5B, 2.5C). Cell wall stress, as expected, also led to a significant increase in the expression of *trxB,* as previously observed (Fig 2.4A, 2.4B); however, the point mutations in the redox-sensing switch had a significantly smaller effect on the induction of *trxB* and other Spx-controlled genes (Fig 2.5B, 2.5C) by fosfomycin compared to diamide. Thus, oxidation of the Spx protein seems to play a more limited role during induction of the regulon by cell wall stress. Strikingly, further analysis of the CxxC

motif showed that cells harboring a Spx mutant protein lacking both cysteines (i.e. $Spx^{C10AC13A}$) displayed reduced induction of Spx-controlled genes compared to Spx^{C10A} in response to cell wall stress, but increased induction in response to diamide (Fig 2.5C). While unexpected, we note that the increased activity of an $Spx^{C10S,C13S}$ double mutant compared to a single Spx^{C10S} mutant has been seen previously (Birch, 2017). These seemingly incongruous results highlight the complexity of the regulation of Spx, and seem to indicate that other factors likely influence Spx activity.

To further explore changes in Spx oxidation during cell wall stress, we used *in vivo* AMS alkylation experiments. This assay relies on the ability of the AMS maleimide group to covalently modify reduced cysteine residues at neutral pH, which results in an increase in the apparent molecular weight of the protein (~0.5 kDa per AMS molecule attached) as observed by SDS-PAGE. Conversely, disulfide bonds and other oxidized thiol species are resistant to modification. In agreement with previous findings, diamide treatment led to the formation of a non-modified Spx protein band (i.e. lower molecular mass band), consistent with the presence of an intramolecular disulfide bond. By contrast, in untreated cells, we observed the presence of two well-defined protein bands: one faint band that corresponds to Spx + 0 AMS, the oxidized species, as well as a dominant protein band that corresponds to Spx + 2 AMS, the reduced species (Fig 2.5D, 2.5E). Thus, in actively growing cells Spx is mostly reduced, although some oxidized protein is present. Treatment with ampicillin, fosfomycin, and vancomycin resulted in a dramatic accumulation of Spx, and this newly synthesized protein was present largely in the reduced form (i.e. Spx + 2 AMS) (Fig 2.5D). Under these conditions, the intensity of the faint band corresponding to oxidized protein was actually decreased. The present evidence thus suggests that in contrast to disulfide stress, the induction of the Spx regulon under cell wall stress is likely driven by the reduced Spx species.

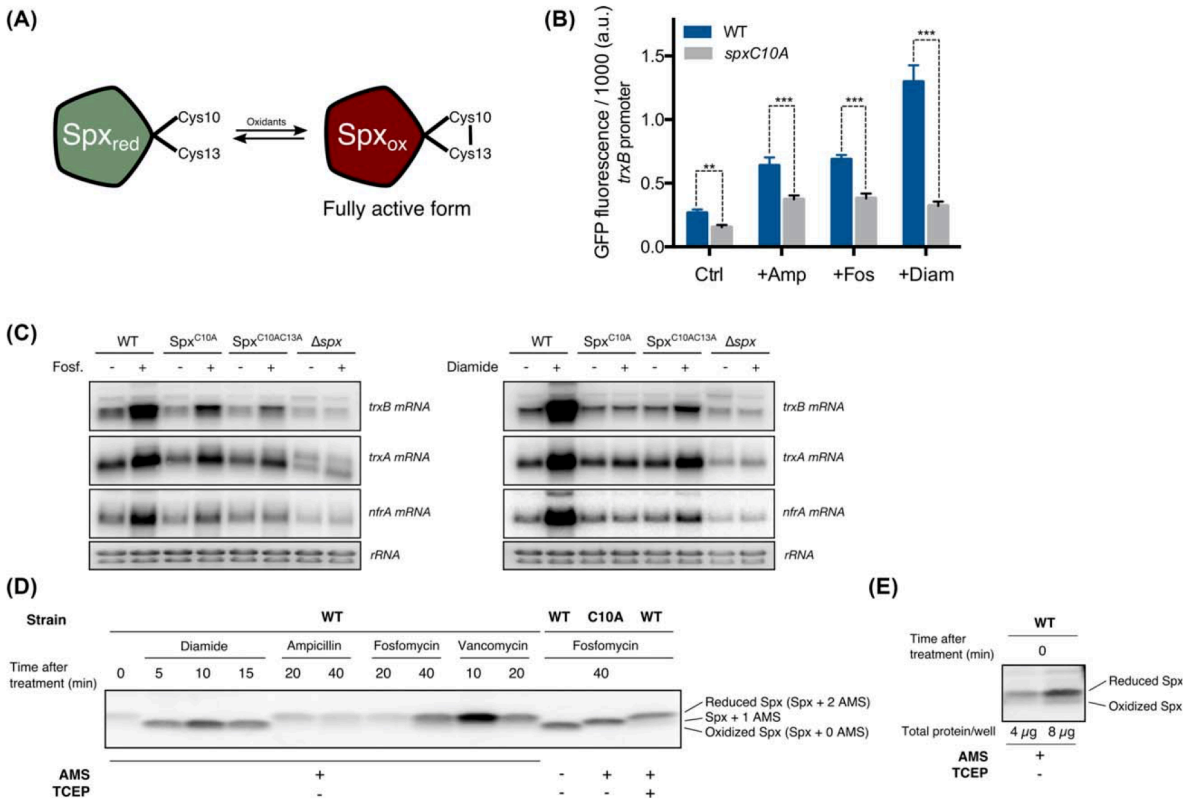

FIG 2.5 ANALYSIS OF THE OXIDATION OF SPX FOLLOWING CELL WALL STRESS.

A) The CXXC redox-sensing switch is located at the N-terminal part of the Spx protein. Formation of a disulfide bond in presence of oxidants such as diamide fully activates the protein. B) A transcriptional fusion of the *trxB* promoter and *gfp* was used to monitor the activity of the Spx and Spx^{C10A} proteins. Fluorescence was measured using flow cytometry. One, two, and three asterisks indicate significant differences with P <0.05, P<0.01, and P<0.001, respectively, as estimated using the T-test. C) Evaluation of the induction of four Spx-dependent genes in response to both fosfomycin (RNA harvested after 40 min. of treatment) and diamide (RNA harvested after 10 min. of treatment) in cells harboring either the *spx*, *spx*^{C10A}, or *spx*^{C10AC13A} allele by using northern blot. D) AMS alkylation experiments were carried out to determine the oxidation state of the Spx protein *in vivo*. Diamide was included as positive control for oxidation of Spx, and vancomycin was included to further state the fact that Spx is present in the reduced state following cell wall stress. Appropriate controls were included to estimate the molecular weight of alkylated Spx with zero, one, or two AMS molecules linked. A total of 4 μg of protein were loaded in each well. The concentrations of antibiotic were used as listed in Fig 2.1B. E) Both protein bands correspond to the same protein samples as in Fig 2.5C at time zero, but overexposed. The amounts listed below the gel correspond to the total amount of protein loaded in each well.

2.3.6 Cells lacking Spx display increased sensitivity towards cell wall active antibiotics

We next set to determine if induction of the Spx regulon in response to cell wall stress increased cell survival in the presence of inhibitors of PG synthesis. Consistent with previous findings (Nakano *et al.*, 2003), cells lacking Spx displayed reduced survival after treatment with diamide (Fig 2.6A). Cells lacking Spx were also more sensitive to cell wall antibiotics, with a dramatic loss of viability within the first 60 min. of treatment (Fig 2.6A); the same time period during which the Spx regulon is induced. Ectopic complementation of *spx* with either the entire *yjbC-spx* operon (Fig 2.6A) or an IPTG-controlled *spx* allele (Fig S1), as expected, restored the wild-type phenotype.

We next asked whether cells lacking the P_{M1} promoter, or those unable to form the disulfide switch, were also more sensitive to the PG inhibitors. For this, we monitored bacterial survival 90 min. after treatment. Cells harboring the mutant P_{M1}* promoter display increased sensitivity to fosfomycin and D-cycloserine, but not ampicillin or diamide. Since the Spx regulon is active throughout exponential phase and slightly induced even in the absence of σ^M control (Fig 2.4C, 2.4D), it is likely that this partial activity suffices to provide ampicillin resistance. Fosfomycin and D-cycloserine resistance, by contrast, required full upregulation of the Spx regulon. As expected, no differences between WT and WT(P_{M1*}) were observed upon diamide challenge (Fig 2.6B), which is consistent with the fact that both strains display similar Spx protein and *trxB* induction levels under disulfide stress (Fig 2.3C, 2.4C). Finally, we note that cells unable to form the disulfide switch (i.e. Spx^{C10A}) were significantly more sensitive to all three antibiotics studied (Fig 2.6B). It is not yet clear if this is indicative of a requirement for Spx oxidation during cell wall stress or if this reflects the fact that these cells display overall reduced Spx activity (Fig 2.5B).

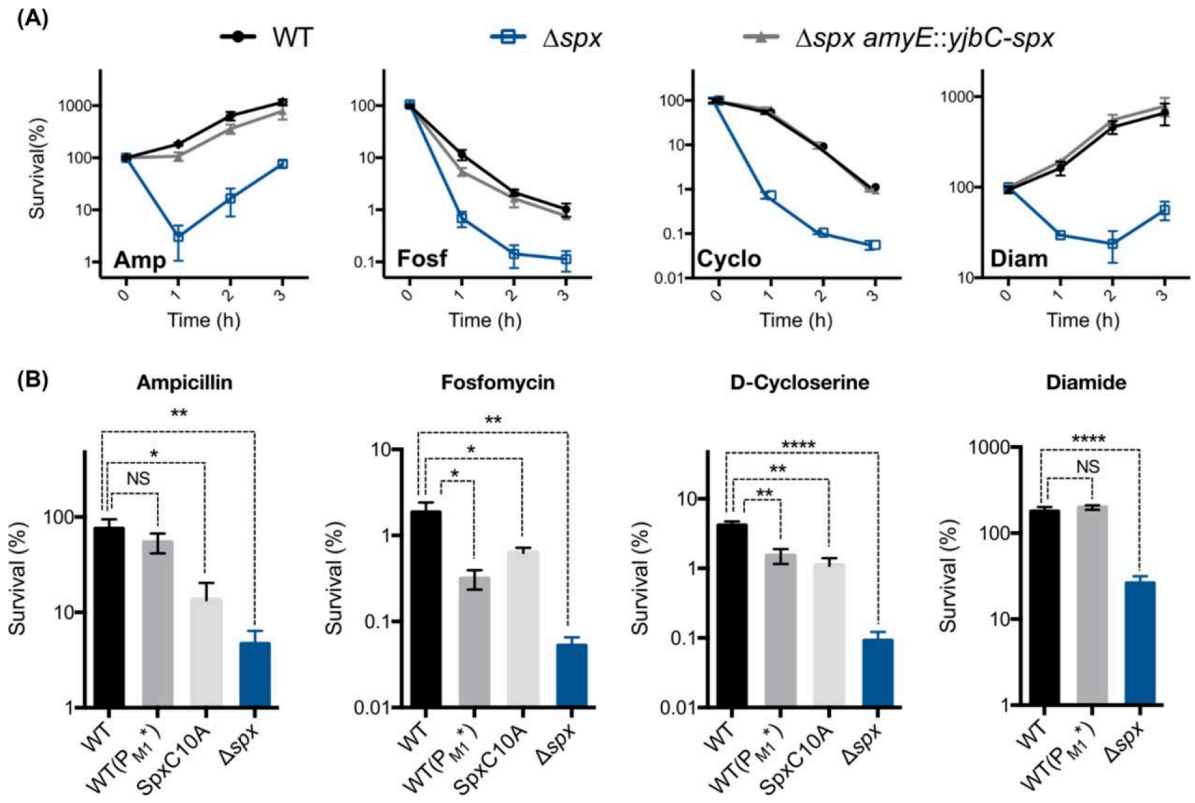

FIG 2.6. CELLS LACKING SPX ARE SENSITIVE TO CELL WALL ANTIBIOTICS.

A) Time-killing experiments in WT, *spx* null mutant, and the complementation strain. Cells were grown until OD_{600} reached ~0.5, and treated with ampicillin, fosfomycin, D-cycloserine, and diamide. Samples were taken each hour and survival was monitored by plating serial dilutions on plain LB plates. B) The survival of cells harboring the P_{M1}* promoter or *spx*^{C10A} allele was measured after 90 min. of ampicillin, fosfomycin, D-cycloserine, and diamide treatment. One, two, and three asterisks indicate significant differences with P<0.05, P<0.01, and P<0.001, respectively, as estimated using the Dunnett Test and comparing the mutant strains against WT. NS indicates no significant differences. The experiments are the result of at least five biological replicates.

2.4 Discussion

Spx controls the expression of a large number of genes in response to disulfide stress (Nakano *et al.*, 2003; Rochat *et al.*, 2012); a stress condition that results in the formation of nonnative disulfide bonds protein in the cytoplasm and therefore leads to protein misfolding and aggregation. Under disulfide stress, activation of the Spx regulon relies on a decrease in Spx proteolysis, as well as oxidation of the Spx redox-sensing switch (Larsson *et al.*, 2007; Zhang and Zuber, 2007; Garg *et al.*,

2009; Engman and von Wachenfeldt, 2015). The decrease in Spx proteolysis occurs due to the aggregation of the adaptor protein YjbH through a mechanism that does not involve its cysteine residues (Engman and von Wachenfeldt, 2015), and a decrease in the activity of the ATP-dependent ClpXP protease (Garg *et al.*, 2009). Other stress conditions that result in formation of protein aggregates, such as heat shock, also cause Spx accumulation (Runde *et al.*, 2014; Engman and von Wachenfeldt, 2015). Remarkably, no changes in the expression of the *spx* gene are generally observed when the cells are treated with diamide (Fig 2.1B), suggesting that the complex transcriptional architecture of the *spx* gene (Fig 2.1A) plays a minimal role in the induction of the regulon under disulfide stress (Rochat *et al.*, 2012).

In most bacteria, the PG layer is essential for growth and development. Due to its essentiality, bacteria possess specific transcription factors that allow cells to respond to physical or chemical threats that can compromise its integrity (Jordan *et al.*, 2008). Among these transcription factors, the ECF σ factor σ^M plays a central role, as it coordinates the expression of genes involved in cell wall biosynthesis and cell division (Helmann, 2016). In addition, σ^M mediates transcriptional induction of the *spx* gene under conditions in which the integrity of the cell wall is compromised. Induction of the *yjbC-spx* operon, for example, has been observed in transcriptomic studies of *B. subtilis* cells treated with vancomycin (Cao *et al.*, 2002b; Eiamphungporn and Helmann, 2008), enduracidin and bacitracin (Rukmana *et al.*, 2009), and ramoplanin and moenomycin (Salzberg *et al.*, 2011). *B. subtilis* protoplasts also display significant *spx* upregulation compared to either walled cells or L-forms (Kawai *et al.*, 2015). We here confirmed the upregulation of *spx* by treatment with early and late PG synthesis inhibitors and further show that the P_{M1} promoter, which is primarily controlled by σ^M, is the major driver of this transcriptional induction (Fig 2.2). Membrane antibiotics that also target the cell envelope did not lead to Spx accumulation (Fig 2.3D). Remarkably, in contrast to disulfide stress, transcriptional induction of *spx* in response to cell wall stress is required for both accumulation of Spx protein (Fig 2.3) and induction of Spx-controlled genes (Fig 2.4).

Cells lacking σ^M are significantly more sensitive to drugs that interrupt essential steps in the construction of PG, which is at least partially due to an inability to induce genes involved in PG homeostasis (Mascher *et al.*, 2007; Luo and Helmann, 2012). Although the roles of many genes in the σ^M regulon have been well defined (Cao *et al.*, 2005; Eiamphungporn and Helmann, 2008; Meeske *et al.*, 2015; Meeske *et al.*, 2016; Zhao *et al.*, 2016), the contributions of each σ^M target operon to antibiotic resistance are incompletely understood. Here, we show that antibiotic-dependent induction of the Spx regulon protects cells against killing by cell wall antibiotics (Fig 2.6). Although *spx* transcription is up-regulated from the P_{M1} promoter upstream of the *yjbC-spx* operon, a clean deletion of the *yjbC* gene does not affect antibiotic resistance (data not shown), and the role of the YjbC protein remains unclear. Our results extend previous findings where artificial induction of Spx conferred high level resistance to the beta-lactam antibiotic cefuroxime (Luo and Helmann, 2012). The Spx-regulated genes that protect against various cell wall antibiotics are not yet known, and likely vary depending on the antibiotic.

The functional role of the redox-sensing switch in the induction of the Spx regulon is complex. *In vitro* an *in vivo* experiments have shown that diamide induction of the canonical Spx-controlled gene *trxB* is largely dependent on the oxidized form of Spx (Nakano *et al.*, 2005; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). The present results support those observations as diamide treatment only led to a comparatively modest increase in *trxB* expression in cells harboring the spx^{C10A} allele (Fig 2.5B, 2.5C). Under cell wall stress, by contrast, several Spx-controlled genes were strongly induced (Fig 2.4A) even when the protein was present primarily in the reduced state (Fig 2.5D); additionally, relatively small differences in induction of *trxB* were observed in cells harboring Spx or Spx^{C10A} (Fig 2.5). Oxidation of the redox-sensing switch, therefore, seems to play a limited contribution in induction of the Spx regulon under cell wall stress. Indeed, it has been previously shown that both reduced and oxidized Spx have similar affinity for the α-CTD domain of RNA polymerase (Nakano *et al.*, 2005).

These results suggest that although induction of *trxB* by diamide requires oxidation of Spx, the sustained high level of reduced Spx seen under cell wall stress conditions also suffices (Fig 2.3).

Interestingly, recent evidence suggests that the contribution of the redox-sensing switch may be promoter specific. Spx-dependent expression of the bacillithiol biosynthesis genes, for example, does not require the formation of an intramolecular disulfide (Gaballa *et al.*, 2013). Furthermore, Rochat et al. (2012) also identified genes that displayed a large variation in their requirement for the oxidized Spx species using *in vitro* transcription. Some genes, for instance, were activated by either oxidized or reduced Spx, whereas other genes were predominantly activated by oxidized Spx. These observations raise the possibility that the Spx regulon under cell wall stress (where Spx is largely reduced) may differ from that observed under diamide treatment (where Spx is largely oxidized).

A link between Spx and the cell wall stress response has been directly or indirectly observed in other Gram-positive species. In *Lactococcus lactis*, for example, deletion of an Spx paralog called SpxB renders the cells sensitive to lysozyme (Veiga *et al.*, 2007); SpxB controls the expression of an *O*-acetylase that modifies the peptidoglycan subunits and protects the cells against lysozyme. In *Staphylococcus aureus*, deletion of YjbH, which causes accumulation of Spx, renders the cells more resistant to diverse cell wall antibiotics (Gohring *et al.*, 2011). In this species, the Spx-dependent induction of the *trfA* gene, a *B. subtilis mecA* homologue, has been associated with increased antibiotic resistance (Jousselin *et al.*, 2013). In *Enterococcus faecalis*, cells lacking Spx display increased sensitivity towards ampicillin and vancomycin, two inhibitors of the synthesis of PG (Kajfasz *et al.*, 2012). Notably, in *B. anthracis* and *S. mutants,* the Spx proteins are also potential regulator of genes involved in PG biosynthesis (Veiga *et al.*, 2007; Barendt *et al.*, 2013), suggesting a direct effect of Spx on cell wall homeostasis. In *B. subtilis*, interestingly, Spx is capable of binding the promoters of *murAA*, *ponA*, and *mreB*, that encode proteins involved in synthesis of PG; their expression, however, is Spx-independent in response to diamide (Rochat *et al.*, 2012) as well as fosfomycin (Fig S2). The conditions in which those genes are induced by Spx remain to be determined. The functional roles of

Spx are broad and not limited to disulfide and cell wall stress; virulence, biofilm formation, and resistance to oxidative stress also require the activity of Spx (Pamp *et al.*, 2006; Veiga *et al.*, 2007; Wang *et al.*, 2010; Kajfasz *et al.*, 2012; Barendt *et al.*, 2013; Zheng *et al.*, 2014).

The results presented here support a model in which cell wall damage leads to the induction of the Spx regulon in a σ^M-dependent fashion. This induction is critical for the cell wall stress response, since cells lacking it are more sensitive than WT against antibiotics interrupting various steps in the peptidoglycan biosynthesis pathway. In response to cell wall stress, two notable differences were observed in the regulation of Spx activity in comparison to diamide. First, σ^M was required for transcriptional induction of the *spx* gene, and therefore induction of the Spx regulon. Second, Spx primarily accumulated in the reduced form and this form sufficed to induce the regulon. It is likely that down-regulation of ClpXP proteolysis also contributes to induction of the Spx regulon under cell wall stress, as suggested by the observation that cells unable to induce P_{M1} still display a modest increase in Spx accumulation and activity. The present results not only expand the conditions known to induce the Spx regulon, but also highlight the diversity of regulatory mechanisms that can lead to induction of Spx.

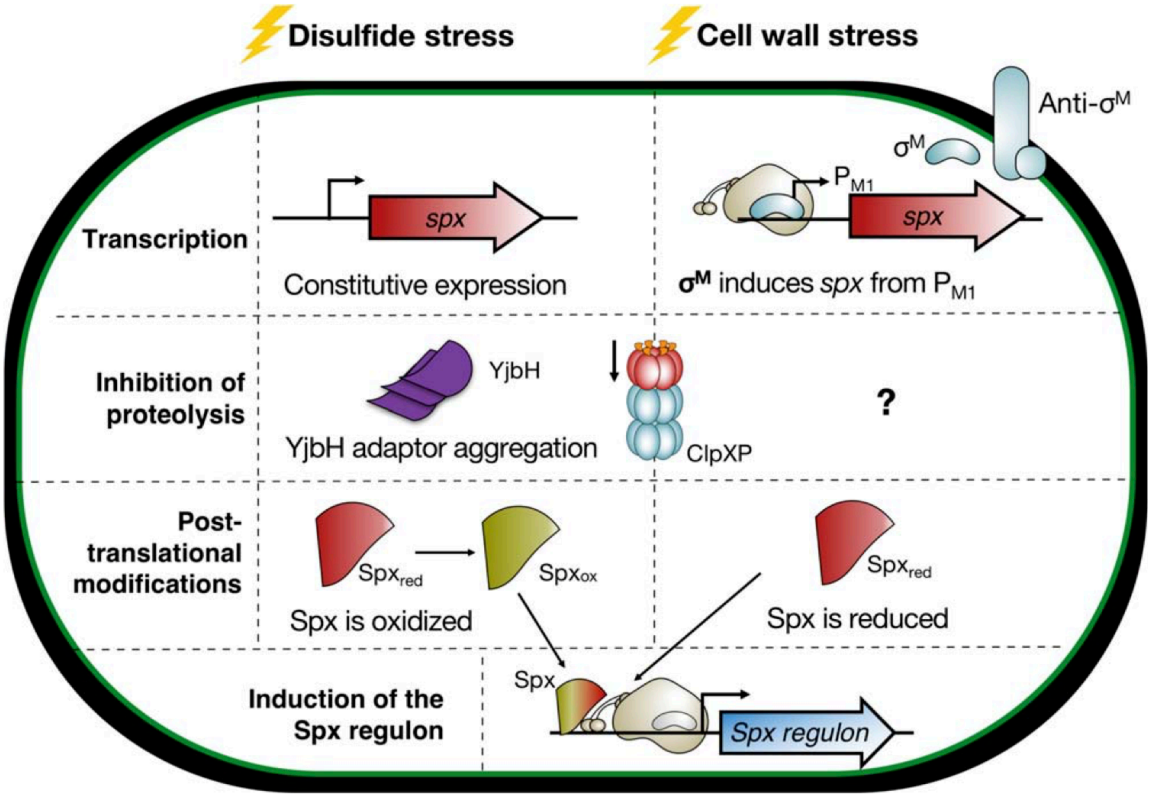

FIG 2.7 MODEL OF SPX REGULATION UNDER DISULFIDE AND CELL WALL STRESS.

Under disulfide stress, the expression of the *spx* gene is constitutive. Reduced proteolysis due to YjbH aggregation and ClpXP oxidation result in Spx accumulation. Disulfide stress then leads to formation of an intramolecular disulfide bond, which results in high levels of induction of the Spx regulon. In comparison, cell wall stress leads to transcriptional induction of the *spx* gene through activation of the P_{M1} promoter in a σ^M-dependent fashion, a process that is required for Spx accumulation. Whether stabilization of Spx occurs under cell wall stress remains under investigation. Spx then accumulates primarily in its reduced form, and activates the Spx regulon. In both cases, induction of the Spx regulon is required for survival against stress.

2.5 Experimental procedures

2.5.1 Bacterial strains and culture conditions

All bacterial strains are listed in Table 2.1. The primers used are listed in Tables S1-S3. *Bacillus subtilis* strains (all based on the *B. subtilis* 168 wild-type) were grown under standard conditions: lysogeny broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) broth at 37 ºC with vigorous shaking, unless otherwise stated. *Escherichia coli* DH5a was used for plasmid construction. Antibiotics were added to the growth medium when appropriate: 100 μg ml^{-1} ampicillin for *E. coli*, and 1 μg ml^{-1} erythromycin plus 25 μg ml^{-1} of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg ml^{-1} chloramphenicol, 100 μg ml^{-1} spectinomycin, 5 μg ml^{-1} tetracycline and 10 μg ml^{-1} kanamycin for *B. subtilis*.

2.5.2 Strain constructions

For construction of the complementation strains, HB18907 and HB18923, the mutagenic primers for P_{M1}* and *spx*^{C10A} were DR150/DR151 and DR84/DR85, respectively (Table S1). The external primers were DR51/DR52, and genomic DNA was used as template. The pGFPStar* vector was amplified using DR28/DR29. The assembled constructs were transformed into an *spx*::*kan* mutant strain. For construction of the GFP promoter-reporter fusions the primers for mutagenesis for the P_{M1}*, P_{M2}*, and P_B* promoters were DR150/DR151, DR152/DR153, and DR154/DR155 (reverse and forward), respectively. The external primers for the insert were DR51/DR181. Genomic DNA was used as template. The vector was PCR amplified using DR29/DR180. For construction of the P_{trxB}-GFP, the *trxB* promoter was PCR amplified from chromosomal DNA using DR132/DR133, and the pGFPStar vector using DR130/DR131. In all cases, the PCR-amplified vectors were digested using DpnI for 1 h at 37ºC. All fragments were then purified and used for Gibson assembly. The ligation

product was transformed into *E. coli* DH5a. Then the plasmids were extracted, and 1 μg of plasmid used to transform *Bacillus subtilis* 168. The insert was verified by PCR and sequencing.

For construction of the *yjbC-spx* mutants at locus (HB18697 and HB18698) we used the same mutagenic primers as for HB18907 and HB18923. Overlap PCR was used to construct the *B. subtilis* strains, which became resistant to kanamycin. Chromosomal DNA was used as template. The external primers for the *yjbC-spx* fragment were DR70/DR81. Then, the fragment containing the mutations was fused with a fragment containing the kanamycin cassette, which was amplified using primers 1295/1296; as well as a DNA region encompassing the *yjbE* and *mecA* genes downstream *yjbC-spx*, which was amplified using DR82/DR71. The ~5 kb fragment was transformed into *B. subtilis*, and confirmed by PCR and sequencing. For construction of a WT strain harboring the kanamycin cassette at the same position, the protocol was identical except for the fact that mutagenic primers were not included.

For construction of the *sigM* complementation strain (HB18646), the *sigM* gene and its promoter were amplified using DR194/DR198, and cloned by Gibson Assembly into pDG1663, which was amplified using DR192/DR193. The chosen primers for the vector removed the *lacZ* gene. The DNA was transformed into HB18640. The *sigW*::*ery* strain was obtained from the Bacillus Genetic Stock Center, and backcrossed into 168.

For construction of the $P_{M2,A}$-*lacZ* and P_A-lacZ fusions, the promoter regions were amplified using DR325/DR327 and DR326/DR327, respectively, and cloned into pDG1663 using the BamH1 and EcoRI restriction sites. The promoters $P_{M2,A}$ and P_A included the regions -170 to +121 and -80 to +121, respectively, relative to the P_A transcription start site as described by Leelakriangsak *et al.* (2007). The vector containing the fusion was transformed into *B. subtilis* 168. The P_{M1}-*spx* fusion at the *amyE* site was constructed using PCR to fuse the region including the P_{M1} promoter and the *spx* coding sequence. For this, the primers DR333/DR334 and DR332/DR335 were used for PCR amplification of the promoter and coding region, respectively. The resulting fragment was cloned into

pDG1662 using the HindIII and EcoRI restriction enzymes. The vector containing the fusion was transformed into HB18905. The inserts and vectors were fused with T4 ligase, and cloned into *E. coli* DH5a. The integrity of all constructs was verified by sequencing.

2.5.3 RNA isolation

Cells were grown under standard conditions until OD_{600} reached ~0.5, and then treated or not with cell wall antibiotics or diamide. RNA was isolated using the hot phenol-chloroform method. Briefly, five ml of cells are collected, centrifuged at 5000 rpm, and resuspended in 400 μl of TE buffer (pH 8.0) supplemented with 20 mg ml^{-1} of lysozyme and 10 μl of proteinase K. The mixture was incubated at room temperature for 10 min., and then transferred to a 1.7 microfuge tube. Next, 23 μl of 2 M sodium acetate buffer (pH 5.2), 45 μl of 10% SDS, and 400 μl of acid phenol was added, the mixture was vigorously vortexed, and the tubes were incubated at 65 ºC for 10 min. Next, the tubes were placed in ice for 5 min., and then centrifuged at top speed for 15 min. at 10ºC. The upper phase was carefully removed, mixed with equal parts of chloroform, and then vortexed to form an emulsion. The mixture was centrifuged at 14000 rpm for 10 min., and then the upper phase was removed. The fraction containing the RNA (~400 μL) was adjusted to 0.3 M sodium acetate buffer (pH 5.2), and mixed with a similar volume of isopropanol for RNA precipitation. The precipitated RNA was centrifuged at 15ºC for 30 min. at 14000 rpm, and the supernatant was carefully decanted. The RNA was washed with chilled 70% ethanol, incubated for 10 min. at RT to dissolve salts, and centrifuged at 14000 rpm at 4 ºC for 10 min. The supernatant was carefully removed, and the samples were air-dried for 10. min at 50ºC. The RNA was resuspended in 70 μl of 1 mM sodium citrate (pH 6.2). RNA concentration and purity was determined using spectrophotometry (Nanodrop), while RNA integrity was checked using an RNA formaldehyde gel.

Table 2.1 Strains used in this study

Strain	Genotype	Reference/Construction
168	*trpC2*	Lab strain
HB18801	*168 spx::kan*	Lab strain
HB18816	*168 spx::kan amyE::P_{spx}-spx-gfp (cm)*	This study
HB18907	*168 spx::kan amyE:: $P_{spx(PM1*)}$-spx-gfp (cm)*	This study
HB18923	*168 spx::kan amyE:: P_{spx}-spxC10A-gfp (cm)*	This study
HB18926	*168 amyE::P_{spx}-GFP (cm)*	This study
HB18650	*168 amyE::$P_{spx(PM1*)}$-GFP (cm)*	This study
HB18651	*168 amyE::$P_{spx(PM2*)}$-GFP (cm)*	This study
HB18652	*168 amyE::$P_{spx(PB)}$-GFP (cm)*	This study
HB18634	*168 amyE::P_{empty}-GFP (cm)*	This study
HB18640	*168 sigM::markerless*	This study
HB18646	*168 sigM::markerless thrC::sigM (ery)*	This study
HB18824	*168 sigW::mls*	BKE strain -> 168
HB18914	*168 sigW::mls sigM::tet*	BKE strain -> *sigM::tet*
HB18696	*168 spx::P_{spx}-spx (kan) amyE::P_{trxB}-gfp (cm)*	This study
HB18697	*168 spx::P_{spx}-spxC10A (kan) amyE::P_{trxB}-gfp (cm)*	This study
HB18698	*168 spx::$P_{spx(PM1*)}$-spx (kan) amyE::P_{trxB}-gfp (cm)*	This study
HB18699	*168 spx::kan amyE::P_{trxB}-gfp (cm)*	This study
HB18900	*168 sigX::spec*	Lab strain
HB18915	*168 sigX::spec sigM::tet*	Lab strain
HB18917	*168 sigX::spec sigM::tet sigW::mls*	Lab strain
HB13551	*168 sigB::cm*	Lab strain
HB23013	*168 spx::$P_{spx(PM1*)}$-spx (kan) amyE::P_{M1}-spx (cm)*	This study
HB23010	*168 thrC::$P_{M2,A}$-lacZ (ery)*	This study
HB23011	*168 thrC::P_{A}-lacZ (ery)*	This study
HB18905	*168 spx::$P_{spx(PM1*)}$-spx (kan)*	This study
HB18805	*168 spx::kan amyE::P_{IPTG}-spx (spec)*	Lab strain

2.5.4 Northern Blot

A total of 1-10 μg of RNA were loaded per lane in an RNA denaturing agarose gel, and separated at 70 V for about 1.5 h. RNA was transferred to a nylon membrane by the downward capillarity technique using Transfer Buffer (Bio-Rad, US), and cross-linked using a UV 302 nm transilluminator (Spectroline, US) for 3 min. The blots were maintained at 4ºC in conical tubes until ready to analyze. Probes were synthesized using *in vitro* transcription and ^{32}P-radiolabeled alpha-UTP. The T7 promoter was included in the reverse primer. Probe size ranged between 150-250 nt. Hybridization was performed at 68ºC overnight using the UltraHyb buffer and following manufacturer's instructions. Then the membrane was washed twice in 2X SSC buffer + 0.1% SDS at 68ºC for 5 min. each, and then twice in 0.1 X SSC buffer + 0.1% SDS at 68ºC for 15 min. The blotted membrane was wrapped in a plastic sheet, and exposed to a phosphorimaging cassette overnight prior to visualization using a Typhoon 7000 machine (GE, US).

2.5.5 Western Blot

Samples were collected before and after treatment with the antibiotics or diamide. A total of 5 ml were collected, washed in PBS, and resuspended in 150 μl of Buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% glycerol) supplemented with complete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche). Then cells were disrupted by sonication, and centrifuged for 15 min. at top speed at 4ºC. The soluble fraction was collected and quantified using the Bradford Assay, with BSA as standard. Reducing sample buffer was added to the protein extract, and 10 μg of protein were loaded in a 4-20% SDS-PAGE. Proteins were transfer onto a PVDF membrane using the TransBlot Turbo Transfer System (Bio-Rad, USA). The membrane was blocked using 5% protein blotting blocker dissolved in TTBS for 1 h at RT. Then, the primary antibodies were resuspended in 0.5% protein blotting blocker dissolved in TTBS and incubated overnight at 4ºC. Finally, an anti-rabbit HRP-

conjugated secondary antibody was added and incubated for 1 h at RT. The membrane was washed four times in TTBS, one in TBS, and then visualized using the Clarity Western ECL substrate (Bio-Rad, USA).

2.5.6 Time-dependent killing assays

The cells were grown until OD_{600} reached ~0.5. Then, cells were treated or not with different chemicals, and incubated at 37ºC with agitation. At specific time points, samples were taken, washed in saline, and serially diluted in 0.15 M NaCl. Ten-*µ*l aliquots of each dilution were plated on LB plates, incubated for 16 h at 28ºC, and the resulting colonies enumerated.

2.5.7 Flow cytometry

The fluorescence of cells harboring the transcriptional fusions with *gfp* was determined using flow cytometry. Briefly, cells were grown until OD_{600} reached ~0.5, and then were mock-treated or treated with ampicillin, fosfomycin, or diamide. Forty-seven minutes after treatment, the cells were collected, washed twice in PBS, and finally resuspended in PBS buffer. Fluorescence was read in a BD FACSAria using a 488 nm excitation laser. A total of ~50,000 cells were sampled per event.

2.5.8 AMS alkylation

To determine the oxidation state of the cysteine residues in Spx, cells were grown under standard conditions until OD_{600} reached ~0.5, and then treated or not with cell wall antibiotics or diamide. At specific time points, 1.8 mL samples were collected and precipitated using 0.2 mL of 100% TCA, and then rapidly incubated on ice. The amount of protein was normalized by resuspending the cells to a final concentration of OD_{600}=1.0. The samples were then washed in ice-cold 100% acetone,

and the pellet was allowed to dry for 10 min. at room temperature. Next, cells were resuspended in 100 μl of denaturing buffer (2% SDS, 1 mM EDTA, 250 mM Tris-HCl, pH 6.8), and then supplemented or not with AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid disodium salt, ThermoFisher) to produce a ~20 fold molar excess. Cells were sonicated until the lysate cleared and the alkylation reaction was incubated in the dark at 37ºC for 2 h. For the fully reduced and alkylated samples, after sonication the samples were pre-treated with TCEP (in a ~20 molar excess), and then alkylated using AMS as described. Non-reducing sample buffer was then added to the alkylation reactions, and 4.0 μg were loaded in a 4-20% SDS-PAGE gel for western blot.

2.5.9 RT-qPCR

Samples were taken as previously described, and the RNA was isolated using the RNeasy Kit (Qiagen) following manufacturer's instructions. The isolated RNA was treated with DNase I for 20 min. at 37 ºC in order to eliminate genomic DNA contamination. Then, RNA was re-extracted using acidic phenol-chloroform followed by ethanol precipitation. The RNA was resuspended in DEPC-treated water, and the purity and quality were verified using a spectrophotometer (Nanodrop). The integrity of the RNA was monitored in native agarose gels. A total of 200 ng of RNA were reverse transcribed using TaqMan™ Reverse Transcription Reagents (Thermo-Fisher Scientific) following manufacturer's instructions. qPCR was carried out using the iTaq Universal SYBR Green Supermix (Bio-Rad) according to the instructions of the manufacturer. Primers for the 23S rRNA and *gyrA* gene were used as reference.

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2.7 References

Antelmann, H., Scharf, C., Hecker, M., and Hecker, M. (2000) Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis. *J Bacteriol* 182: 4478–4490.

Barendt, S., Lee, H., Birch, C., Nakano, M.M., Jones, M., and Zuber, P. (2013) Transcriptomic and phenotypic analysis of paralogous *spx* gene function in *Bacillus anthracis* Sterne. *Microbiologyopen* 2: 695–714.

Birch, C. (2017) Redox Control and Mechanism of Spx-activated Transcription in *Bacillus subtilis*. Scholar Archive. 3945. http://digitalcommons.ohsu.edu/etd/3945/

Cao, M., Kobel, P.A., Morshedi, M.M., Wu, M.F.W., Paddon, C., and Helmann, J.D. (2002a) Defining the *Bacillus subtilis* σ^W regulon: A comparative analysis of promoter consensus search, run-off transcription/macroarray analysis (ROMA), and transcriptional profiling approaches. *Journal of Molecular Biology* 316: 443–457.

Cao, M., Moore, C.M., and Helmann, J.D. (2005) *Bacillus subtilis* paraquat resistance is directed by σ^M, an extracytoplasmic function sigma factor, and is conferred by YqjL and BcrC. *J Bacteriol* 187: 2948–2956.

Cao, M., Wang, T., Ye, R., and Helmann, J.D. (2002b) Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. *Mol Microbiol* 45: 1267–1276.

Czarny, T.L., Perri, A.L., French, S., and Brown, E.D. (2014) Discovery of novel cell wall-active compounds using P_{ywaC}, a sensitive reporter of cell wall stress, in the model gram-positive bacterium *Bacillus subtilis*. *Antimicrob Agents Chemother* 58: 3261–3269.

Eiamphungporn, W., and Helmann, J.D. (2008) The *Bacillus subtilis* σ^M regulon and its contribution to cell envelope stress responses. *Mol Microbiol* 67: 830–848.

Engman, J., and von Wachenfeldt, C. (2015) Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. *Mol Microbiol* 95: 51–63.

Gaballa, A., Antelmann, H., Hamilton, C.J., and Helmann, J.D. (2013) Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx. *Microbiology* 159: 2025–2035.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009) The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *J Bacteriol* 191: 1268–1277.

Gohring, N., Fedtke, I., Xia, G., Jorge, A.M., Pinho, M.G., Bertsche, U., and Peschel, A. (2011) New role of the disulfide stress effector YjbH in beta-lactam susceptibility of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 55: 5452–5458.

Guariglia-Oropeza, V., and Helmann, J.D. (2011) *Bacillus subtilis* σ^V confers lysozyme resistance by activation of two cell wall modification pathways, peptidoglycan O-acetylation and D-alanylation of teichoic acids. *J Bacteriol* 193: 6223–6232.

Hastie, J.L., Williams, K.B., Bohr, L.L., Houtman, J.C., Gakhar, L., and Ellermeier, C.D. (2016) The Anti-sigma Factor RsiV Is a Bacterial Receptor for Lysozyme: Co-crystal Structure Determination and Demonstration That Binding of Lysozyme to RsiV Is Required for σ^V Activation. *PLoS Genet* 12: e1006287.

Helmann, J.D. (2016) *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Curr Opin Microbiol* 30: 122–132.

Ho, T.D., Hastie, J.L., Intile, P.J., and Ellermeier, C.D. (2011) The *Bacillus subtilis* extracytoplasmic function σ factor σ^V is induced by lysozyme and provides resistance to lysozyme. *J Bacteriol* 193: 6215–6222.

Jervis, A.J., Thackray, P.D., Houston, C.W., Horsburgh, M.J., and Moir, A. (2007) SigM-Responsive Genes of *Bacillus subtilis* and Their Promoters. *J Bacteriol* 189: 4534–4538.

Jordan, S., Hutchings, M.I., and Mascher, T. (2008) Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol Rev* 32: 107–146.

Jousselin, A., Kelley, W.L., Barras, C., Lew, D.P., and Renzoni, A. (2013) The *Staphylococcus aureus* thiol/oxidative stress global regulator Spx controls *trfA*, a gene implicated in cell wall antibiotic resistance. *Antimicrob Agents Chemother* 57: 3283–3292.

Kajfasz, J.K., Mendoza, J.E., Gaca, A.O., Miller, J.H., Koselny, K.A., Giambiagi-deMarval, M., *et al.* (2012) The Spx Regulator Modulates Stress Responses and Virulence in *Enterococcus faecalis*. *Infect Immun* 80: 2265–2275.

Kajfasz, J.K., Rivera-Ramos, I., Abranches, J., Martinez, A.R., Rosalen, P.L., Derr, A.M., *et al.* (2010) Two Spx Proteins Modulate Stress Tolerance, Survival, and Virulence in *Streptococcus mutans*. *J Bacteriol* 192: 2546–2556.

Kawai, Y., Mercier, R., Wu, L.J., Domínguez-Cuevas, P., Oshima, T., and Errington, J. (2015) Cell growth of wall-free L-form bacteria is limited by oxidative damage. *Curr Biol* 25: 1613–1618.

Larsson, J.T., Rogstam, A., and von Wachenfeldt, C. (2007) YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. *Mol Microbiol* 66: 669–684.

Leelakriangsak, M., and Zuber, P. (2007) Transcription from the P_3 promoter of the *Bacillus subtilis spx* gene is induced in response to disulfide stress. *J Bacteriol* 189: 1727–1735.

Leelakriangsak, M., Kobayashi, K., and Zuber, P. (2007) Dual negative control of *spx* transcription initiation from the P_3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *J Bacteriol* 189: 1736–1744.

Lin, A.A., Walthers, D., and Zuber, P. (2013) Residue substitutions near the redox center of *Bacillus subtilis* Spx affect RNA polymerase interaction, redox control, and Spx-DNA contact at a conserved cis-acting element. *J Bacteriol* 195: 3967–3978.

Luo, Y., and Helmann, J.D. (2012) Analysis of the role of *Bacillus subtilis* σ^M in beta-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol Microbiol* 83: 623–639.

Mascher, T., Hachmann, A.-B., and Helmann, J.D. (2007) Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. *J Bacteriol* 189: 6919–6927.

Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekelanos, J.J., Kahne, D., *et al.* (2016) SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* 537: 634–638.

Meeske, A.J., Sham, L.-T., Kimsey, H., Koo, B.-M., Gross, C.A., Bernhardt, T.G., and Rudner, D.Z. (2015) MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. *Proc Natl Acad Sci USA* 112: 6437–6442.

Nakano, S., Erwin, K.N., Ralle, M., and Zuber, P. (2005) Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol* 55: 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A.D., and Zuber, P. (2003) Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proc Natl Acad Sci USA* 100: 13603–13608.

Nakano, S., Zheng, G., Nakano, M.M., and Zuber, P. (2002) Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. *J Bacteriol* 184: 3664–3670.

Newberry, K.J., Nakano, S., Zuber, P., and Brennan, R.G. (2005) Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. *Proc Natl Acad Sci USA* 102: 15839–15844.

Pamp, S.J., Frees, D., Engelmann, S., Hecker, M., Ingmer, H. (2006) Spx Is a Global Effector Impacting Stress Tolerance and Biofilm Formation in *Staphylococcus aureus*. *J Bacteriol* 188: 4861–4870.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinova, A., Korelusova, J., Leduc, A., *et al.* (2012) Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. *Nucleic Acids Res* 40: 9571–9583.

Rukmana, A., Morimoto, T., and Takahashi, H. (2009) Assessment of transcriptional responses of *Bacillus subtilis* cells to the antibiotic enduracidin, which interferes with cell wall synthesis, using a high-density tiling chip. *Genes Genet Syst* 84: 253–267.

Runde, S., MoliEre, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., *et al.* (2014) The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *Bacillus subtilis*. *Mol Microbiol* 91: 1036–1052.

Salzberg, L.I., Luo, Y., Hachmann, A.-B., Mascher, T., and Helmann, J.D. (2011) The *Bacillus subtilis* GntR family repressor YtrA responds to cell wall antibiotics. *J Bacteriol* 193: 5793–5801.

Veiga, P., Bulbarela-Sampieri, C., Furlan, S., Maisons, A., Chapot-Chartier, M.-P., Erkelenz, M., *et al.* (2007) SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. *J Biol Chem* 282: 19342–19354.

Wang, C., Fan, J., Niu, C., Wang, C., Villaruz, A.E., Otto, M., and Gao, Q. (2010) Role of Spx in biofilm formation of *Staphylococcus epidermidis*. *FEMS Immunol Med Microbiol* 59: 152–160.

Wecke, T., Bauer, T., Harth, H., Mäder, U., and Mascher, T. (2011) The rhamnolipid stress response of *Bacillus subtilis*. *FEMS Microbiol Lett* 323: 113–123.

Zhang, Y., and Zuber, P. (2007) Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. *J Bacteriol* 189: 7669–7680.

Zhao, H., Sun, Y., Peters, J.M., Gross, C.A., Garner, E.C., and Helmann, J.D. (2016) Depletion of Undecaprenyl Pyrophosphate Phosphatases Disrupts Cell Envelope Biogenesis in *Bacillus subtilis*. *J Bacteriol* 198: 2925–2935.

Zheng, C., Xu, J., Li, J., Hu, L., Xia, J., Fan, J., *et al.* (2014) Two Spx Regulators Modulate Stress Tolerance and Virulence in *Streptococcus suis* Serotype 2. *PLoS One* 9: e108197–13.

Zuber, P. (2009) Management of oxidative stress in Bacillus. *Annu Rev Microbiol* 63: 575–597.

2.8 Supplementary information

2.8.1 Supplementary Figures

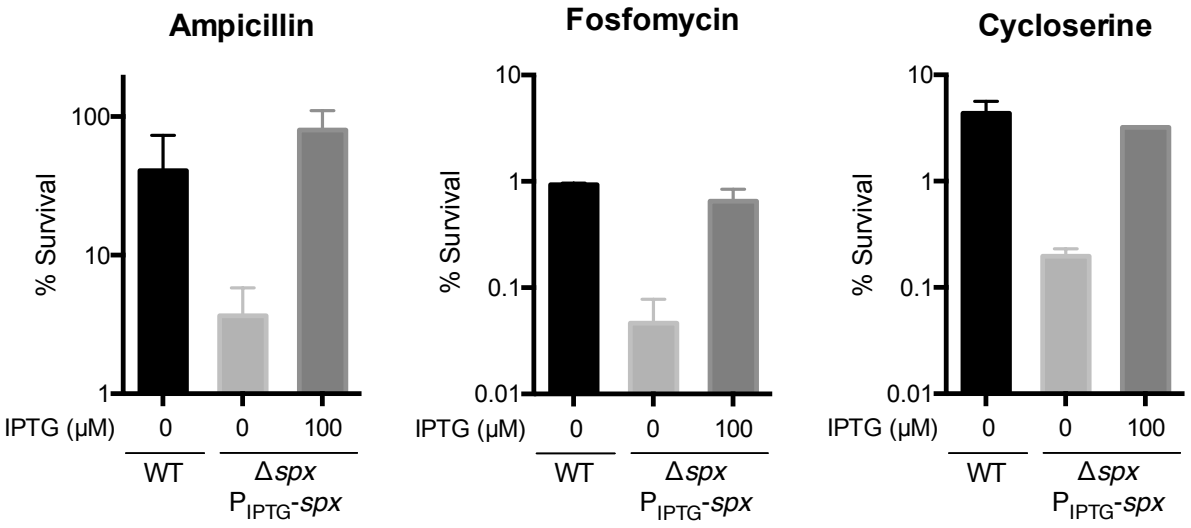

Fig S1 Complementation of an Δ*spx* mutant with an IPTG-inducible *spx* allele restored the resistance phenotype against cell wall antibiotics. Briefly, cells were grown up to mid-exponential phase in the presence or absence of IPTG (100 μM), and then challenged with ampicillin (2 μg/ml), fosfomycin (200 μg/ml), and D-cycloserine (250 μg/ml). The number of viable cells were quantified after 90 min. of incubation. Error bars indicate SEM.

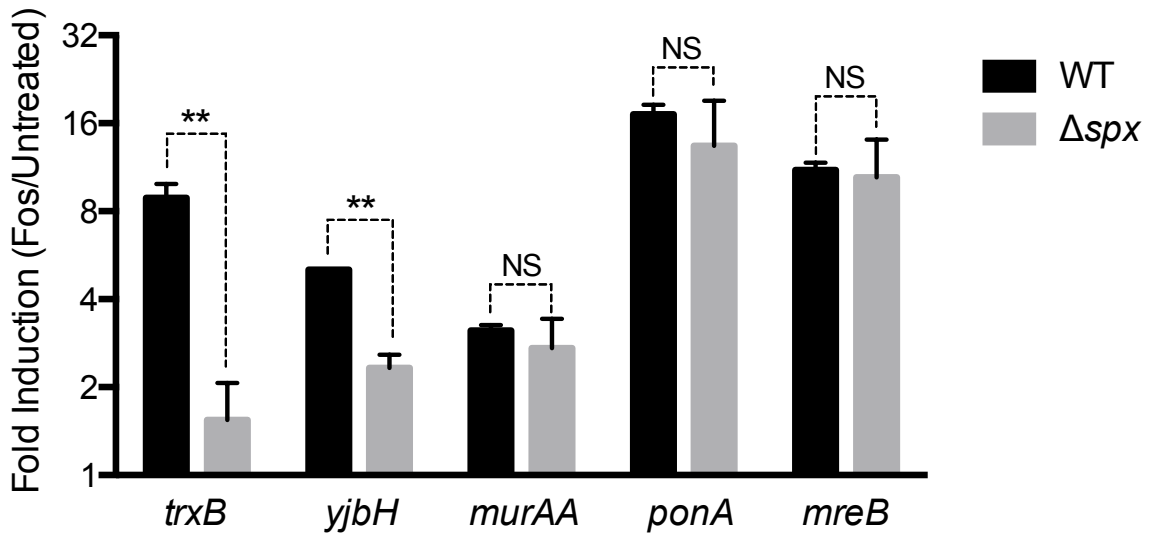

Fig S2 Induction of Spx-controlled and putatively Spx-controlled genes was studied by RT-qPCR in WT and Δ*spx* strains. As observed, the known Spx-controlled genes *trxB* and *yjbH* were significantly upregulated by fosfomycin (after 40 min. of treatment), and the induction was Spx-dependent. The putatively controlled genes *murAA*, *ponA*, and *mreB* were significantly induced by fosfomycin, but no differences were found between WT and Δ*spx*. One, two, and three asterisks indicate significant differences with $P<0.05$, $P<0.01$, and $P<0.001$, respectively, as estimated using the T-Test and comparing WT and Δ*spx*. NS indicates no significant differences.

2.8.2 Supplementary Tables

Table S1 Primers used in this study

Strain	Genotype
DR70 yjbC-spx Fwd	GTCGTTTGCGAGTATAGCAGC
DR71 yjbC-spx Rev	CGTCAAGATCCTCAGATGCTGG
DR81	TCACCTCAAATGGTTCGCTGAAGAAGGCTGATGACTCAGC
DR82	GCCTACGAGGAATTTGTATCGTTATTTTTCAACCTGCTGGT
DR150 Pspx PM1 Fwd	GGAAGTTCGCACAAGTTTATAGATGTAAG
DR151 Pspx PM1 Rev	CTTACATCTATAAACTTGTGCGAACTTCC
DR152 Pspx PM2 Fwd	AAAAATGTCTTTATCCAGCAAATTTTTAATG
DR153 Pspx PM2 Rev	ATTTGCTGGATAAAGACATTTTTTTCTTAGG
DR154 Pspx PB Fwd	ATATAAAACATTTCTTCTTGTTTAAACAGC
DR155 Pspx PB Rev	CAAGAAGAAATGTTTTATATCTAAAAGTATG
DR181 Pspx Rep Rev	GCATGGTATTTTCCTCCTTTTCATCTTCACTCCTCTAATTAG
DR51 yjbC-spx Fwd2	CCCGGGAAGGAGGAACTACTCCCCGTCTGGTTCATTACAG
DR52 yjbC-spx Rev2	GTTCTTCTCCTTTACGCATGGTATTTTCCTCCTTTATTCTTTTGATGATACGATC
DR28 pGFPStar Fwd	ATGCGTAAAGGAGAAGAACTTTTC
DR29 pGFPStar Rev	AGTAGTTCCTCCTTCCCGGGAAAG
DR84 SpxC10A Fwd	CATCACCAAGCGCTACTTCATGCAGAAAGG
DR85 SpxC10A Rev	GAAGTAGCGCTTGGTGATGTGTATAG
DR130 GFP Fwd	GGCTTTCCCGGGAAGGAG
DR131 GFP Rev	CGCGGTAAAATGACTGTAAAGG
DR132 PtrxB Fwd	CTTTACAGTCATTTTACCGCGTACAAAACAAATGGACACGTAGG
DR133 PtrxB Rev	CTCCTTCCCGGGAAAGCCCCAACAACGACAAGTTCTTTGC
DR192 pDG1663 Fwd	TAATAACCGGGCAGGCCATG
DR193 pDG1663 Rev	AGTAGTTCACCACCTTTTCCC
DR194 sigM Fwd	GGAAAAGGTGGTGAACTACTAGGCCGATTCTGGAGGCTTC
DR198 sigM Rev	CATGGCCTGCCCGGTTATTACTGGTCGCTCATTTCCCCA

DR180 pGFPStar Fwd 2	AAAGGAGGAAAATACCATGCG
#1295 Kan Fwd	CAGCGAACCATTTGAGGTGATAGG
#1296 Kan Rev	CGATACAAATTCCTCGTAGGCGCTCGG
DR325 PA+PM2 spx	ATCAGAATTCGAGGTCTTGGAAATCATCGATG
DR326 PA spx	ATCAGAATTCGGATTGACACATTTTTTTCTTAGGATACTG
DR327 PA+PM2 Rev	ATCAGGATCCCATGAAGTACAGCTTGGTGATG
DR332 spx comp fwd	ATCAGAATTCGCGATTTTGACTCCTTGGTCAG
DR333 spx comp Rev	ATCGAAGCTTGATTGTCAGAGACAAAAAGCTGTC
DR334 DelProm Spx Fwd	ATGTTCATCCTACTAATTAGAGGAG
DR335 DelProm Spx Rev	CCTCTAATTAGTAGGATGAACATCTTGTTTAAACAGCCTTACATCTATAAAGAC

Table S2 Primers used for RNA probes

Strain	**Genotype**
spx fwd	CCAAGCTGTACTTCATGCAGAAAGGC
spx rev	atcgaTAATACGACTCACTATAGGGAGAGATCAAGCGGTACAAATCTTGCAGC
trxB fwd	GCGTGCCTGGCGAAAAAGAATTGG
trxB rev	atcgaTAATACGACTCACTATAGGGAGAGAGCACGGAGTTTATCACGTCTGTG
trxA fwd	AGACTTCTGGGCTCCTTGGT
trxA rev	atcgaTAATACGACTCACTATAGGGAGAGCGCTTCTTTTGGTTTGAAG
yjbH fwd	ACGTGCTTTTGGAGATTGCT
yjbH rev	atcgaTAATACGACTCACTATAGGGAGACTTCATCCTCATGCTGCGTA
nfrA fwd	GCTTTTCGGACTGGCAGTAG
nfrA rev	atcgaTAATACGACTCACTATAGGGAGAAAATACGTACGCGGCTTTTG
lacZ fwd	GAGAATCCGACGGGTTGTTA
lacZ rev	atcgaTAATACGACTCACTATAGGGAGAAAATTCAGACGGCAAACGAC

Table S3 Primers used for RT-qPCR

Strain	Genotype
trxB fwd	GCGTGCCTGGCGAAAAAGAATTGG
trxB rev	GAGCACGGAGTTTATCACGTCTGTG
sigM fwd	TGATCACAGCAAAGTAAAGCCCTGGC
sigM rev	CAGGACTTTGAACAGCATTTTGAAAGAGG
sigW fwd	GCTACCGTATGCTTGGCAATGTGC
sigW rev	GTACCAGCCACCTCTGCGTCG
sigX fwd	ACAGGATGTCAGGGATCAGC
sigX rev	CCTCTGTGTTGGGTCGTTTT
yjbH fwd	GATGAAAACGTGCTTTTGGAGATTGCTG
yjbH rev	CTTTAAGCCCTTCATCCTCATGCTGC
murAA fwd	GCTCCTACACTTGGGAAGTCCAG
murAA rev	GCAAGAGTTGCACTTCCGGG
ponA fwd	TCGTGATTGGGACCGCAAATATCTAGG
ponA rev	CCCACCGAAACCGCCAATAGAATAGG
mreB fwd	GTAACGTCTCAGTCAATCCGTGTAGCC
mreB rev	GTTGTCGGATTCTTCAGGAGCTTCTG

CHAPTER III: Stabilization of *Bacillus subtilis* Spx under cell wall stress requires an anti-adaptor protein

The present Chapter is a modified version of the already published manuscript:

Rojas-Tapias, D.F., and Helmann, J.D. (2018b). Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. PLoS Genet *14*, e1007531.

3.1 Summary

Spx is a global transcriptional regulator present in low-GC Gram-positive bacteria, including the model bacterium *Bacillus subtilis* and various human pathogens. In *B. subtilis*, activation of Spx occurs in response to disulfide stress. We recently reported, however, that induction of Spx also occurs in response to cell wall stress, and that the molecular events that result in its activation under both stress conditions are mechanistically different. Here, we demonstrate that, in addition to up-regulation of *spx* transcription through the alternative sigma factor σ^M, full and timely activation of Spx-regulated genes by cell wall stress requires Spx stabilization by the anti-adaptor protein YirB. YirB is itself transcriptionally induced under cell wall stress, but not disulfide stress, and this induction requires the CssRS two-component system, which responds to both secretion stress and cell wall antibiotics. The *yirB* gene is repressed by YuxN, a divergently transcribed TetR family repressor, and CssR~P acts as an anti-repressor. Collectively, our results identify a physiological role for the YirB anti-adaptor protein and show that induction of the Spx regulon under disulfide and cell wall stress occurs through largely independent pathways.

3.2 Introduction

In its natural habitat, the soil-dwelling bacterium *Bacillus subtilis* is continuously exposed to stressful conditions that can compromise its survival. To adapt, bacteria must be able to sense the stress and respond accordingly. Adaptation to stress often requires the interplay of multiple signaling pathways and regulators. At the transcriptional level, gene expression is controlled by modulation of the activity of transcription factors, which through precise molecular interactions redirect the activity of RNA polymerase at specific sets of genes (Browning and Busby, 2016). In *B. subtilis*, for example, the cell envelope stress response is mediated by the individual or coordinated action of extracytoplasmic (ECF) sigma factors (e.g. σ^M, σ^W, and σ^X) (Helmann, 2016), two-component signal transduction systems (e.g. LiaRS and BceSR) (Jordan *et al.*, 2008), and other transcription regulators (e.g. Spx) (Jordan *et al.*, 2008; Helmann, 2016; Rojas-Tapias and Helmann, 2018). The activity of transcription factors can be regulated by changes in their expression or allosteric regulation of their activity. Adaptation to stress may also involve regulated proteolysis of transcription factors (Kirstein *et al.*, 2009).

Proteolysis mediated by the Clp ATP-dependent proteases plays a critical role in regulation, as it permits the selective degradation of specific sets of proteins (Kirstein *et al.*, 2009). The proteins degraded by the Clp proteases generally contain a protein tag, which is recognized by either the protease itself or an adaptor and targets them for degradation (Kirstein *et al.*, 2009). When degradation requires an adaptor, the synthesis of an anti-adaptor protein can antagonize its activity, and allow the stabilization of the target protein (Bougdour *et al.*, 2006; Bougdour *et al.*, 2008; Battesti and Gottesman, 2013). In *B. subtilis*, for example, the proteolysis of the master regulator of competence ComK requires the adaptor protein MecA and the ClpCP protease. The presence of ComS, an anti-adaptor protein, allows ComK accumulation by interfering with the MecA-ComK interaction (Battesti and Gottesman, 2013). Also, in *Escherichia coli* and *Salmonella*, a set of anti-adaptors expressed

under various environmental conditions (e.g. phosphate starvation, DNA damage, or magnesium starvation) permits stabilization, against ClpXP-mediated proteolysis, of the sigma factor RpoS through direct interaction with the adaptor RssB (Bougdour *et al.*, 2006; Bougdour *et al.*, 2008).

The Spx protein is a global regulator in *Bacillus subtilis,* and other low-GC Gram-positive bacteria (Phylum *Firmicutes*) (Nakano *et al.*, 2003; Pamp *et al.*, 2006; Veiga *et al.*, 2007; Turlan *et al.*, 2009). Induction of the Spx regulon is best understood in the case of disulfide stress, but it is also noted under conditions that result in protein denaturation and misfolding (i.e. heat shock or ethanol stress) (Nakano *et al.*, 2003; Runde *et al.*, 2014). Recently, cell wall stress was also reported to trigger the induction of the Spx regulon (Rojas-Tapias and Helmann, 2018). Spx controls the expression of a large number of genes that help the cells to cope with stressful conditions, and includes genes involved in the synthesis of cysteine and bacillithiol, as well as the thioredoxin system, and the ATP-dependent Clp proteases (Nakano *et al.*, 2003; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). While the functional role of the Spx regulon during disulfide and heat stress is fairly well understood (Nakano *et al.*, 2003; Runde *et al.*, 2014), its role during cell wall stress is less clear.

A complex regulatory network drives the expression, stability, and activity of Spx. At the transcriptional level, the expression of *spx* is driven from at least three promoters controlled by different sigma factors: σ^B, σ^M, and σ^A (Antelmann *et al.*, 2000; Leelakriangsak and Zuber, 2007; Eiamphungporn and Helmann, 2008; Rojas-Tapias and Helmann, 2018). The induction of the σ^M-dependent promoter (i.e. P_{M1}) is required for activation of the Spx regulon in response to cell wall stress (Rojas-Tapias and Helmann, 2018), whereas expression of *spx* from the intergenic promoters is sufficient to complement an *Δspx* knockout mutant for diamide resistance (Nakano *et al.*, 2003; Leelakriangsak and Zuber, 2007). The functional role of the σ^B promoter in the induction of the Spx regulon has not yet been defined. Additionally, the protein repressors PerR and YodB modulate the expression of *spx* in response to hydrogen peroxide and electrophilic compounds, respectively (Leelakriangsak *et al.*, 2007). The activity of Spx is modulated by a redox-sensing switch (i.e. contains

a CxxC motif) located at its N-terminus (Nakano *et al.*, 2005), which increases the activity of the protein when oxidized. Oxidation of Spx is, however, not required for the induction of all Spx-regulated genes (Rochat *et al.*, 2012; Gaballa *et al.*, 2013; Rojas-Tapias and Helmann, 2018), and thus the requirement for Spx oxidation seems to depend on the specific nature of the stress. It is still unknown, however, the extent to which the oxidation status of Spx impacts the composition of the regulon.

Although *spx* is highly transcribed in exponentially growing cells, Spx levels remain low due to active proteolysis (Nakano *et al.*, 2002). Spx degradation occurs upon binding of the adaptor protein YjbH to a region near the Spx C-terminus, which targets the protein for degradation via the ATP-dependent protease ClpXP (Larsson *et al.*, 2007; Garg *et al.*, 2009; Chan *et al.*, 2014). Under disulfide stress, the oxidation of YjbH and ClpX, as well as the aggregation of YjbH, result in a dramatic reduction in Spx proteolysis (Larsson *et al.*, 2007; Garg *et al.*, 2009; Engman and Wachenfeldt, 2015): accumulation of Spx, along with the oxidation of its redox switch, then lead to activation of the regulon. Accumulation of Spx under cell wall stress, by contrast, largely depends on transcriptional up-regulation of *spx*, although post-transcriptional effects also appear to play a role (Rojas-Tapias and Helmann, 2018).

In this study, we demonstrate that, in addition to transcriptional induction of *spx* by an alternative sigma factor (i.e. σ^M) (Rojas-Tapias and Helmann, 2018), stabilization of Spx is also required for full induction of the Spx regulon in response to cell wall stress. Interestingly, this stabilization is mediated by an anti-adaptor protein (i.e. YirB), which is rapidly induced under conditions of cell wall stress but not disulfide stress. The expression of *yirB* itself is regulated by the coordinated action of both a two-component system (i.e. CssRS) and a TetR-like repressor (i.e. YuxN). Notably, we found that CssR~P activates the *yirB* promoter by acting as an anti-repressor of YuxN-mediated repression. Finally, we show that activation of the Spx regulon by cell wall stress and disulfide stress takes place through largely independent pathways, providing an example of orthogonality in signal

transduction pathways. This study, therefore, expands the diversity of regulatory mechanisms that govern the induction of the Spx regulon in response to stress.

3.3 Results

3.3.1 A post-transcriptional event contributes to activation of Spx in response to cell wall stress

Previously, we demonstrated that, unlike disulfide stress, induction of the Spx regulon in response to cell wall stress is driven by upregulation of the *spx* gene through a σ^M-dependent promoter (i.e. P_{M1}) (Rojas-Tapias and Helmann, 2018). Consistent with this, cells harboring a non-functional P_{M1} (i.e. P_{M1}*) promoter display a dramatic decrease in both Spx accumulation and induction of Spx-controlled genes in response to cell wall active antibiotics (Rojas-Tapias and Helmann, 2018). Over the course of those experiments, however, we noted that even under conditions wherein *spx* cannot be induced, cell wall stress still led to a slight increase in the concentration of Spx and upregulation of Spx-controlled genes (Rojas-Tapias and Helmann, 2018). This suggested that Spx stabilization may also contribute to induction of the Spx regulon.

To further define if stabilization is important under cell wall stress, we studied Spx accumulation in cells with conditional expression of *spx* from an IPTG-inducible promoter (i.e. P_{hs}-*spx*) (Fig 3.1A). Since these cells are unable to induce *spx* transcription in response to cell wall antibiotics, an increase in Spx levels might reflect protein stabilization. As seen in the wild-type strain (Rojas-Tapias and Helmann, 2018), treatment with different cell wall antibiotics elicited Spx accumulation in the conditional strain (Fig 3.1B), while the *spx* mRNA levels were not elevated upon antibiotic treatment (Fig 3.1C). Induction of the Spx-controlled gene *trxB* was also observed in response to fosfomycin and vancomycin, but not ampicillin (Fig 3.1C). In WT, induction of *trxB* in response to ampicillin is maximal after 20 min of treatment (Rojas-Tapias and Helmann, 2018), which likely explains why no induction

was observed here. Nevertheless, these results further support our hypothesis that cell wall stress results in increased Spx activity independent of transcriptional induction. Altogether, these observations suggest that both transcriptional and post-transcriptional mechanisms are important for induction of Spx-controlled genes in response to cell wall stress.

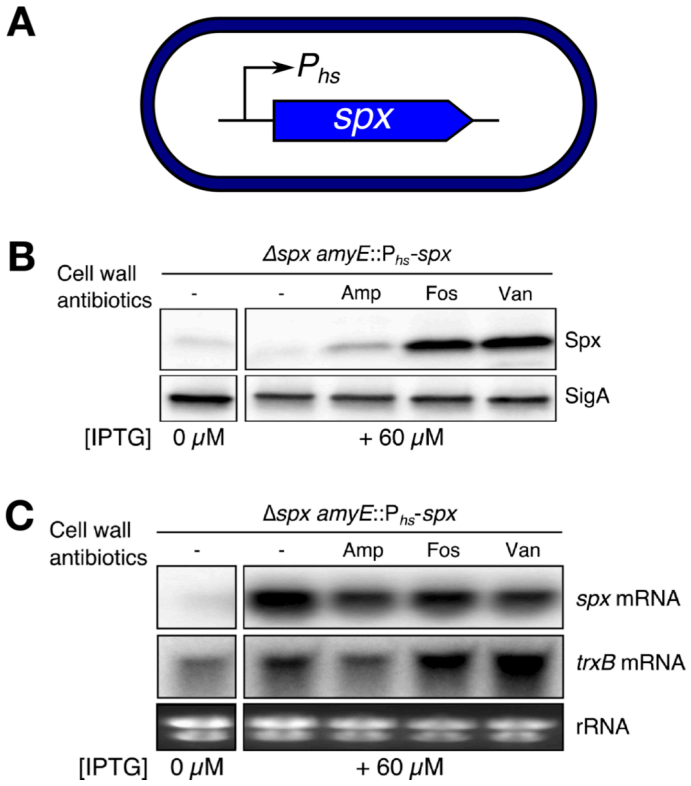

FIG 3.1 A POST-TRANSCRIPTIONAL EVENT CONTRIBUTES TO ACTIVATION OF SPX IN RESPONSE TO CELL WALL STRESS.

A) The *spx* gene was placed under control of IPTG as the only source of *spx* for the cells. B) WT cells were grown with a fixed concentration of inducer and treated or not with 2 μg ml^{-1} ampicillin, 200 μg ml^{-1} fosfomycin, or 1 μg ml^{-1} vancomycin. Spx protein levels were monitored before and after 30 min of treatment using western blot. (C) The *spx* mRNA and *trxB* mRNA levels were simultaneously studied using northern blot. The blots are representative of at least two biological replicates. The "-" symbol indicates untreated cells.

3.3.2 The anti-adaptor protein YirB is required for Spx stabilization

When overexpressed, the YirB protein functions as an anti-adaptor protein that can inhibit the YjbH adaptor resulting in stabilization of Spx (Kommineni *et al.*, 2011). However, the physiological role

of YirB has not been defined, and it is not important under diamide stress conditions (Kommineni *et al.*, 2011). We thus hypothesized that YirB is responsible for stabilization of Spx in response to cell wall stress. To determine if YirB affects Spx accumulation, we measured Spx protein levels in both WT and *ΔyirB* cells in response to vancomycin stress. Consistent with our hypothesis, deletion of YirB caused a decrease in the overall Spx levels, as well as a change in the dynamics of Spx accumulation: while in the WT strain the Spx protein was rapidly accumulated, cells lacking YirB displayed a significant delay in the accumulation of Spx (Fig 3.2A).

In the absence of YirB, Spx accumulated to generally lower levels (Fig 3.2A). This decrease in Spx levels was reflected in reduced expression of the Spx-dependent target gene *trxB* in response to vancomycin treatment (Fig 3.2B). As expected, ectopic complementation of the *yirB* null mutation restored the wild-type phenotype (Fig 3.2B). In the course of these studies, we also observed that cells lacking YirB displayed overall reduced *trxA* and *trxB* promoter activity during growth in LB medium (Fig 3.2C), suggesting that YirB also affects basal expression of Spx-controlled genes in growing cells. As expected, the impact of the deletion of *yirB* on both *trxA* and *trxB* was eliminated in cells lacking the adaptor protein YjbH (Fig 3.2D).

Since YirB is a putative anti-adaptor protein (Kommineni *et al.*, 2011), we reasoned that cells lacking YirB should display reduced Spx stability. To test this idea, we treated log phase cells with vancomycin, incubated the cells for 10 min to allow accumulation of Spx (and potentially YirB), and monitored protein half-life in chloramphenicol treated cells by western blot (Fig 3.2E, 3.2F). Under these conditions, the half-life of Spx in WT was ~2 min, whereas in Δ*yirB* the half-life was reduced to < 1 min (Fig 3.2D, 3.2E). Importantly, the decrease in the stability of Spx in Δ*yirB* cells was not due to abnormally elevated YjbH levels. Indeed, we observed that deletion of YirB led to slightly lower levels of YjbH after 10 min of induction (i.e. when the Spx chase was carried out) (Fig 3.2G), which is consistent with the fact that *yjbH* is itself an Spx-controlled gene (Rochat *et al.*, 2012; Rojas-Tapias and Helmann, 2018).

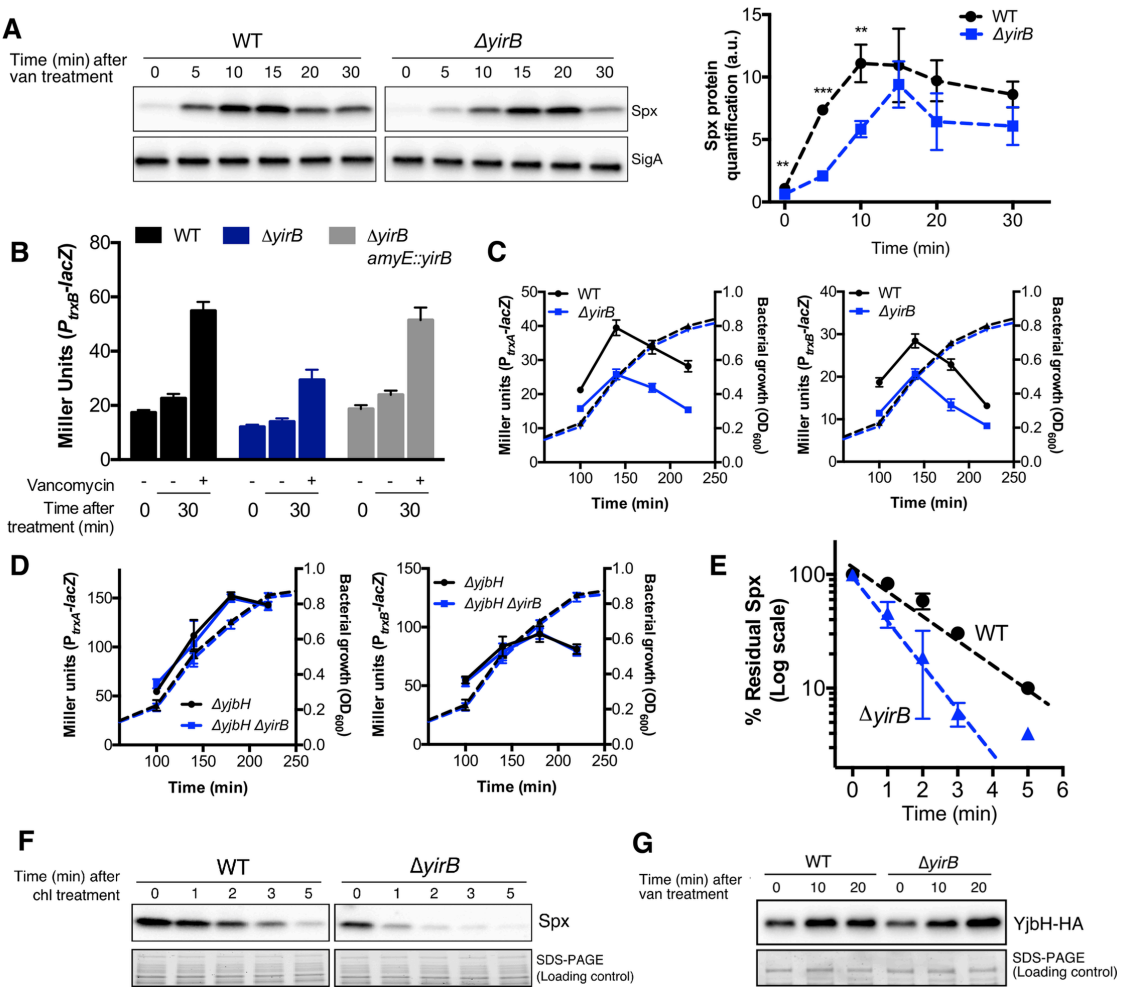

FIG 3.2 THE ANTI-ADAPTOR YIRB IS REQUIRED FOR SPX STABILIZATION.

A) Accumulation of Spx in response to vancomycin treatment was determined in a time-course experiment in WT and *ΔyirB* cells using western blot. A representative blot is shown in the left panel. Relative quantification of Spx protein levels in both strains is plotted on the right panel. Data were normalized using the Spx levels of the WT strain before induction as reference. Error bars represent SEM of three biological replicates. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, as estimated using the T-test. The statistical analysis to compare Spx levels in both strains was independently performed for every time point. B) Expression levels (i.e. β-galactosidase activity) of the Spx-controlled gene *trxB* in WT, *ΔyirB*, and complementation strain (*ΔyirB + amyE::yirB)* following treatment or not with 1 μg ml^{-1} vancomycin. C) Analysis of the activity of the *trxA* and *trxB* promoters in WT and *ΔyirB* during exponential and early stationary phase in LB medium (solid lines). The bacterial growth curves are also displayed on the figure (dashed lines) D) Analysis of the activity of the *trxA* and *trxB* promoters in the strains *ΔyjbH* and *ΔyjbH ΔyirB*. E)- F) Effect of YirB on Spx stability during cell wall stress. The half-life of Spx was determined in exponentially growing cells treated or not with vancomycin. The percentage of remaining Spx was normalized with respect to time 0 min. G) The concentration of YjbH-HA was determined by western blot in WT and Δ*yirB* cells. All experiments were performed in triplicate. Error bars indicate SEM.

3.3.3 YirB also stabilizes Spx in cells with conditional expression of spx

The *spx* gene in under exceptionally complex control, since several promoters (i.e. P_A, P_{M1}, P_{M2}, and P_B) (Antelmann *et al.*, 2000; Leelakriangsak and Zuber, 2007; Eiamphungporn and Helmann, 2008; Rojas-Tapias and Helmann, 2018) and repressors (i.e. PerR and YodB) (Leelakriangsak *et al.*, 2007) modulate its expression. Induction of *spx* in response to cell wall stress, for instance, is driven from P_{M1} as shown in Fig 3.3A (right box). In order to separate any potential effects of YirB on *spx* transcription and determine the actual contribution of YirB to Spx accumulation, we studied Spx dynamics in engineered WT and Δ*yirB* cells featuring conditional expression of *spx* (Fig 3.3A). When cells were grown in presence of a fixed concentration of inducer (i.e. LB medium + 60 μM IPTG; Fig 3.3A, scenario i), we observed that accumulation of Spx occurred in a vancomycin-dependent fashion (Fig 3.3B, left two panels), as previously seen (Fig 3.1B). Deletion of YirB reduced, but did not completely eliminated, the vancomycin-induced accumulation of Spx (Fig 3.3B). Deletion of YirB also affected induction of *trxB* (Fig S1A). These results suggest that YirB-dependent stabilization of Spx (Fig 3.2D, 3.2E) is important for accumulation of Spx and activation of its regulon in response to cell envelope stress.

Under cell wall stress the expression of *spx* is dynamic since the P_{M1} promoter is induced in response to antibiotic treatment (Fig 3.3A, right box). To assess the contribution of YirB under conditions wherein *spx* is induced, we studied the effect of artificial induction of *spx* on Spx levels in the engineered WT and Δ*yirB* cells (Fig 3.3A, scenario ii). For this, cells were grown in LB broth + 20 μM IPTG (i.e. basal induction levels) and then treated with inducer to reach 60 μM IPTG, thereby mimicking the effect of antibiotic induction of *spx* from the P_{M1} promoter. In the absence of vancomycin, addition of inducer resulted in only a transient accumulation of Spx (Fig 3.3C, first panel), and this effect is independent of YirB. This suggests that induction of *spx* from P_{hs} is sufficient for transient accumulation of Spx protein, and the concomitant induction of the σ^M regulon is not required. However, much stronger and long-lasting induction of Spx was observed if there was both an increase in *spx*

transcription and antibiotic treatment (Fig 3.3C, left two panels). Deletion of *yirB* resulted in minor differences between WT and Δ*yirB* under unstressed conditions; however, cell wall stress resulted in increased Spx accumulation and *trxB* induction in a YirB-dependent fashion (Fig 3.3C, S1B). Altogether, these results show that YirB stabilizes Spx under cell wall stress, and that activation of *spx* transcription and Spx stabilization are additive. Further, they suggest that stabilization of Spx can still occur in a YirB-independent fashion.

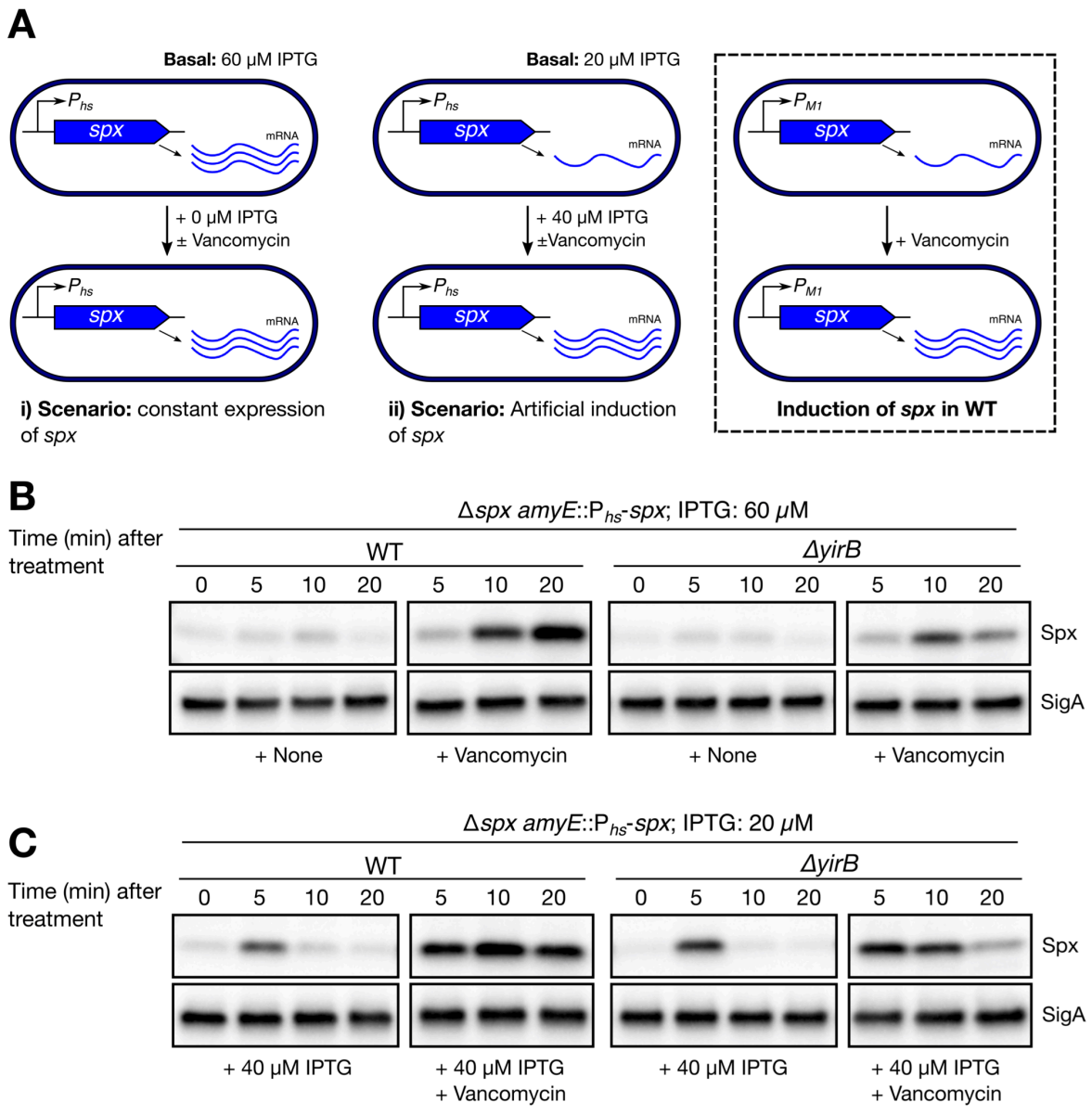

FIG 3.3 YIRB ALSO STABILIZES SPX IN CELLS WITH CONDITIONAL EXPRESSION OF SPX.

(A) Graphical description of the experimental scenarios in Fig 3.3B and 3.3C. The regulation of Spx in wild-type cells was included for reference (right box). (B) Spx levels were monitored in WT and Δ*yirB* cells featuring conditional expression of *spx* (i.e. *spx* expression level was fixed using 60 μM IPTG, scenario i) using western blot. Cells were treated or not with 1 μg ml^{-1} vancomycin. (C) Spx levels were monitored in WT and Δ*yirB* cells featuring conditional expression of *spx* (i.e. *spx* expression was fixed using 20 μM IPTG, scenario ii) using western blot. At the same time, expression of *spx* was upregulated by adding inducer to achieve 60 μM IPTG and cells were treated or not with 1 μg ml^{-1} vancomycin. The blots presented are representative of three biological replicates, which produced similar results.

3.3.4 Both induction of spx and Spx stabilization are required for full and timely induction of Spx-controlled genes in response to cell wall stress

While accumulation of Spx in response to disulfide stress relies on reduced proteolysis (Larsson *et al.*, 2007; Garg *et al.*, 2009; Engman and Wachenfeldt, 2015), Spx accumulation in response to cell wall stress is more complex as it requires both σ^M-dependent *spx* upregulation (Rojas-Tapias and Helmann, 2018) and YirB-mediated Spx stabilization. To study first how both transcriptional induction and stabilization together contribute to activation of the Spx regulon, we monitored the induction of the *trxA* and *trxB* genes using *lacZ* transcriptional fusions in WT, Δ*yirB*, P_{M1*}, and Δ*yirB* P_{M1*} cells. Deletion of *yirB* or inactivation of P_{M1} (i.e. P_{M1*}) led to a significant decrease in the induction of both fusions. Furthermore, in the Δ*yirB* P_{M1*} double mutant the *trxA* and *trxB* genes were no longer responsive to vancomycin treatment (Fig 3.4A, 3.4B). Assessment of the protein levels also provided evidence of additivity, which was more noticeable early after induction (Fig 3.2A, Fig 3.4C). These results demonstrate that both transcriptional induction and stabilization are required for full induction of Spx-controlled genes in response to cell wall stress. They also show that the previously observed induction of Spx-regulated genes in absence of P_{M1} (Rojas-Tapias and Helmann, 2018) was due to YirB.

Our time course studies (Fig 3.2A) suggested that YirB is important for the rapid accumulation of Spx early after antibiotic treatment. In support of this, we also noted that cells lacking YirB, yet still able to induce P_{M1}, display a delay in induction of *trxA* and *trxB* in response to vancomycin treatment (Fig 3.4A, 3.4B). Furthermore, using RT-qPCR, we noticed that whereas the expression of *trxB* and *yjbH* (i.e. two Spx-controlled genes) is strongly and rapidly induced with maximal expression 10 min after treatment, σ^M-dependent induction of *spx* and the autoregulated *sigM* gene is not maximal until 20 min after treatment (Fig 3.4D). The observed dynamics of both accumulation of Spx and induction of Spx-controlled genes therefore reflect both protein stabilization by YirB (most important early after vancomycin treatment) and increased transcription of *spx* (most important at later times).

3.3.5 Induction of the Spx regulon in response to cell wall stress and disulfide stress takes place through independent pathways

Our current and previous findings (Rojas-Tapias and Helmann, 2018) suggest that induction of the Spx regulon in response to both cell wall stress and disulfide stress may occur through fully independent pathways. First, transcriptional induction of *spx* is only important under cell wall stress conditions (Rojas-Tapias and Helmann, 2018); second, unlike disulfide stress, the redox-sensing switch plays a limited role in induction of Spx-controlled genes in response to cell wall-active antibiotics (Rojas-Tapias and Helmann, 2018); and third, YjbH aggregation, which is critical for Spx accumulation under disulfide stress, seems to play no role in Spx stabilization under vancomycin treatment (Fig S2), instead the anti-adaptor protein YirB stabilizes Spx against proteolysis (Fig 3.2, 3.3). To further determine whether the activation of the Spx regulon occurs through independent pathways, we studied the induction dynamics of *trxA* and *trxB* in response to disulfide stress in cells lacking *yirB* (and/or P_{M1}) (Fig 3.4A, 3.4B). Remarkably, in cells treated with diamide, deletion of YirB (or P_{M1}, as expected (Rojas-Tapias and Helmann, 2018)) had no effect on induction of both *trxA* and *trxB*. Likewise,

inactivation of both YirB and P_{M1} had no effect on the responsiveness of both fusions to disulfide stress (Fig 3.4A, 3.4B). We noted, however, a slight decrease in the induction of *trxA* in the *ΔyirB* P_{M1}* strain (Fig 3.4A). Altogether, the present evidence suggests that activation of Spx in response to disulfide and cell wall stress takes place through orthogonal pathways (Fig 3.4A, 3.4B).

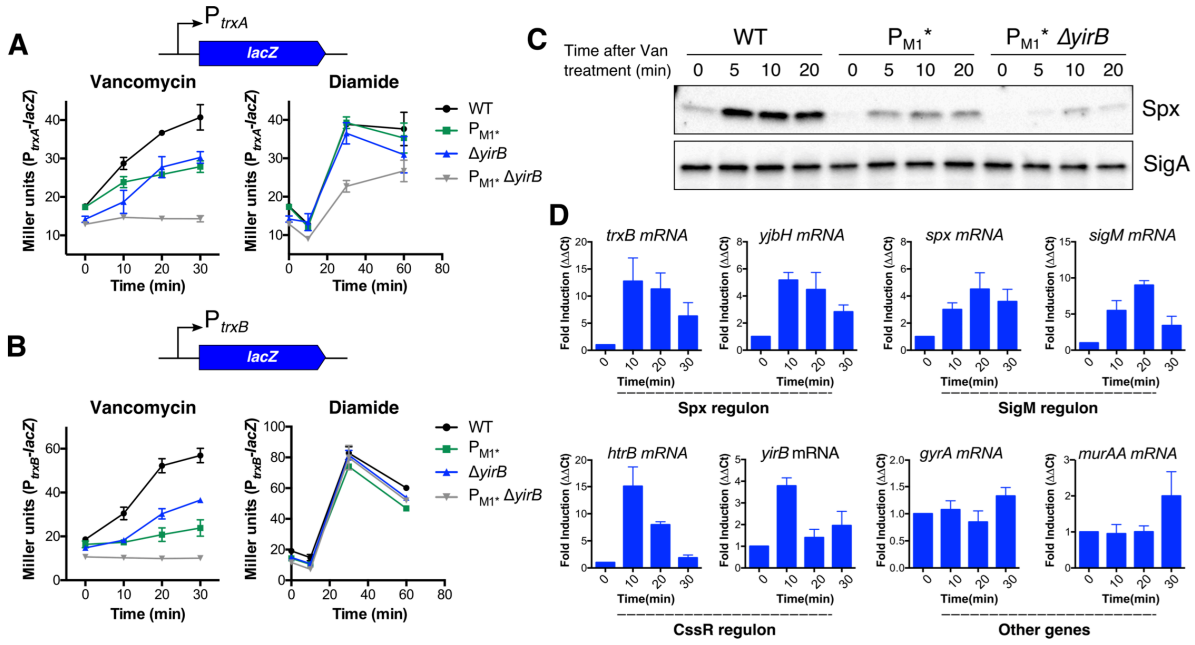

FIG 3.4 BOTH INDUCTION OF SPX AND SPX STABILIZATION ARE REQUIRED FOR FULL AND TIMELY INDUCTION OF SPX-CONTROLLED GENES IN RESPONSE TO CELL WALL STRESS. ACTIVATION OF SPX BY CELL WALL AND DISULFIDE STRESS TAKES PLACE THROUGH INDEPENDENT PATHWAYS.

The expression dynamics of the genes A) *trxA* and B) *trxB* were monitored in WT, *P_{M1}*, ΔyirB,* and the double mutant *P_{M1}* ΔyirB.* Cells were treated with vancomycin or diamide (N=4). C) Spx levels in WT, *P_{M1}*,* and *P_{M1}* ΔyirB* after treatment with vancomycin revealed additivity between transcriptional induction of *spx* and Spx stabilization (N=4), as determined using western blot. D) Expression profile of selected genes using RT-qPCR (N=3). The mRNA levels were normalized against the 23S rRNA. Error bars represent SEM.

3.3.6 Vancomycin induces yirB through the CssRS two-component system

We next sought to determine how YirB activity might itself be regulated. First, we monitored *yirB* mRNA levels under conditions known to induce the Spx regulon including vancomycin (cell wall

stress), diamide (disulfide stress), and ethanol treatment (Nakano *et al.*, 2003; Engman and Wachenfeldt, 2015). Remarkably, only vancomycin treatment resulted in a significant induction of *yirB*, which suggests that the role of YirB is specific to the cell wall stress response (Fig 3.5A). This may also explain why no differences in induction of the Spx regulon were previously found between WT and *ΔyirB* under disulfide stress (Kommineni *et al.*, 2011).

The *yirB* gene is located downstream of *cssRS* and divergently transcribed from *yuxN* (see below). The *cssRS* genes encode a two-component system (TCS) that is known to respond to secretion stress (Hyyryläinen *et al.*, 2001), and *yuxN* encodes a putative repressor in the TetR family with yet unknown activity. We reasoned that since *yirB* and *cssRS* are genetic neighbors, the CssRS TCS might regulate *yirB* under cell wall stress. This hypothesis is supported by the fact that up-regulation of two CssRS-controlled genes (*htrA* and *htrB*) has been previously noted in response to cell wall stress (Cao *et al.*, 2002; Wecke *et al.*, 2011). Moreover, a global transcriptomic study of *B. subtilis* cells growing under a variety of conditions showed that the expression of *yirB* is highly correlated with *htrB*, which is a divergently transcribed gene regulated by the *cssRS* TCS (Nicolas *et al.*, 2012). Consistent with our hypothesis, cells lacking CssR were unable to induce *yirB* upon treatment with vancomycin; ectopic complementation of CssR fully restored the WT phenotype (Fig 3.5B). Furthermore, point mutations that replaced the conserved aspartic acid in the phosphorylation site of CssR by the amino acid alanine (i.e. $CssR^{D52A}$) completely prevented induction of *yirB* following vancomycin treatment, further suggesting that the CssRS two-component system is responsible for upregulation of *yirB* in response to cell wall stress (Fig 3.5C). Since YirB seems to be most important for the increased accumulation of Spx early after antibiotic stress (Fig 3.4), we hypothesized that the CssRS system would be induced rapidly after antibiotic challenge. Indeed, both *yirB* and *htrB* mRNAs accumulate rapidly after vancomycin challenge (Fig 3.4D, Fig 3.5).

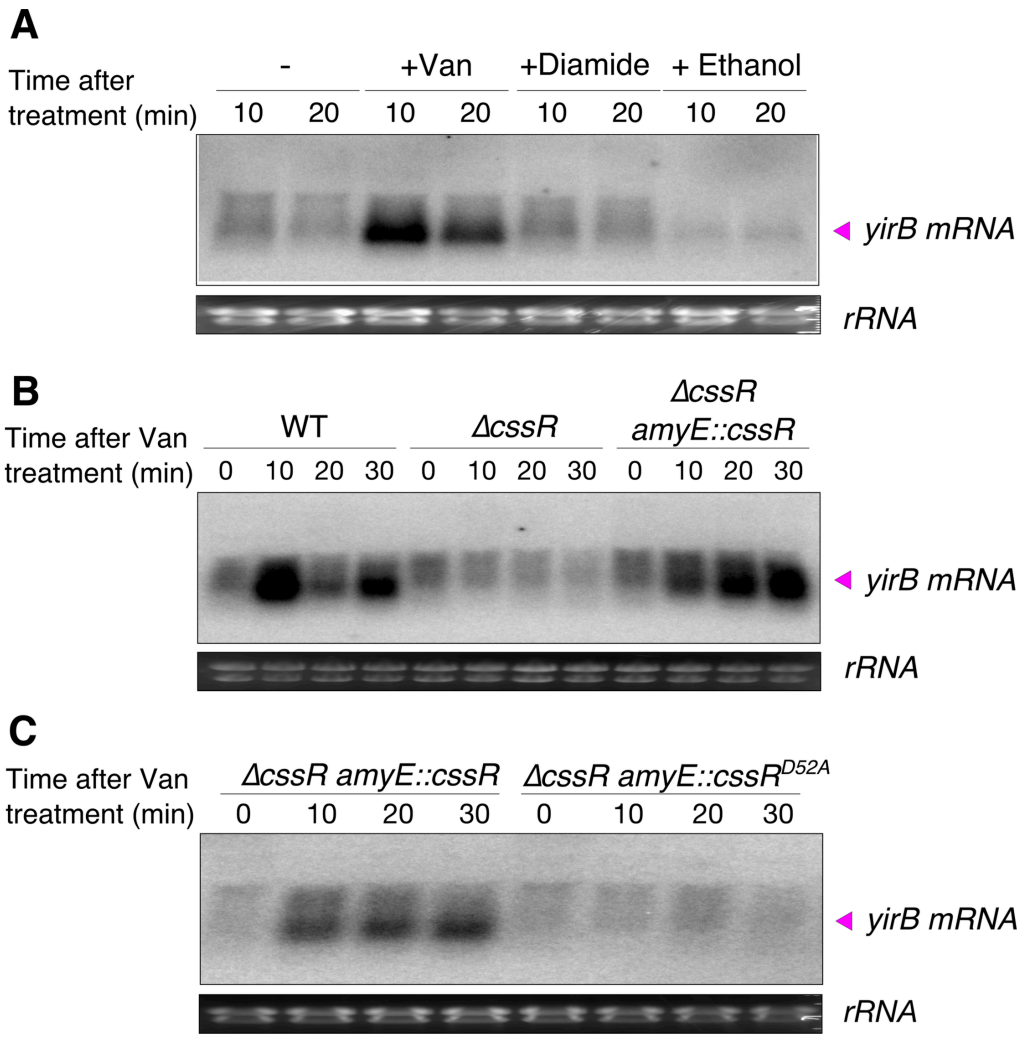

FIG 3.5 VANCOMYCIN INDUCES YIRB THROUGH THE CSSRS TWO-COMPONENT SYSTEM.

A) The *yirB* transcript (~300 nt) is induced under cell wall stress, but not disulfide or ethanol stress. Total RNA was isolated from WT cells treated or not with 1 μg ml^{-1} vancomycin, 500 μM diamide, and 5% ethanol, and *yirB* mRNA levels studied by northern blot. The "-" symbol indicates untreated cells. B) Induction of *yirB* requires the two-component system CssRS. Transcriptional profile of *yirB* in WT, *ΔcssR,* and the complementation strain (*ΔcssR + amyE::cssR*) after treatment with 1 μg ml^{-1} vancomycin as determined by northern blot. C) Induction of *yirB* by CssR, under vancomycin-induced stress, requires the phosphorylation of the Asp52 residue. The expression of *yirB* was studied by northern blot in Δ*cssR* cells complemented ectopically with either *cssR* or *cssR*^{D52A} from the *cssR* native promoter. The presented blots are representative of three independent experiments.

3.3.7 CssR~P induces yirB expression by antagonizing YuxN repression

To further characterize the role of CssR in regulation of *yirB* we isolated RNA from vancomycin treated cells and used 5' RACE to define the transcription start site. Transcription initiates 51 nt upstream of the start codon and two putative CssR boxes (Darmon *et al.*, 2002) were apparent just upstream of the -35 region (Fig 3.6A, 3.6B, S3, S4). We first used promoter truncations (i.e. $P_{yirB(x)}$-*yirB*) to monitor the effect of upstream sequences on *yirB* mRNA levels (Fig 3.6B, 3.6C). Interestingly, truncations that contained either only the RNA polymerase (RNAP) binding-site [i.e. $P_{yirB(-40)}$] or both the RNAP binding-site and putative CssR BoxII [i.e. $P_{yirB(-57)}$] displayed high *yirB* mRNA basal levels and were unresponsive to cell wall stress. The inclusion of predicted BoxI [i.e. $P_{yirB(-122)}$ and $P_{yirB\ (-538)}$] sufficed to restore the WT phenotype: a low basal level and induction by vancomycin (Fig 3.6B). Next, we introduced point mutations to disrupt the most conserved positions in BoxI or BoxII. Point mutations in BoxI rendered *yirB* mRNA basal levels high and unresponsive to vancomycin, while point mutations in the predicted CssRS BoxII had little effect (Fig 3.6D). The activity of *trxB* was also affected by the mutations in P_{yirB} (Fig S5).

Since CssR is required for induction of *yirB* (Fig 3.5), we initially hypothesized that CssR would bind to CssR BoxI to activate transcription. However, our promoter truncation analysis reveals that P_{yirB} is highly active in cells in which BoxI is deleted (Fig 3.6B, 3.6C), and this is supported by the effect of point mutations (Fig 3.6D). These observations imply that BoxI is itself a negative regulatory element for P_{yirB} activity. Analysis of this DNA region in *B. subtilis*, as well as other *Bacillus* species (Fig S4), revealed the presence of a conserved palindromic sequence that lies on top of the predicted CssR BoxI (Fig 3.6B, S4). Likewise, we noted that a similar palindromic region is present in between positions +6 and +28 relative to P_{yirB}; this palindrome overlapped the divergent *yuxN* promoter (Fig S4). These observations suggest a model in which YuxN, a TetR-like repressor, binds to this palindromic sequence as a repressor, both for *yirB* and its own transcription. The role of CssR in this

system could then be as an anti-repressor to alleviate the YuxN-dependent repression by binding to the overlapping CssR BoxI.

To test this model, we explored the role of YuxN in regulating expression of both *yirB* and *yuxN*. For this, we first studied whether YuxN binds the palindromic sequences. We reasoned that if YuxN binds the palindromes, deletion of YuxN would have no effect on expression from constructs lacking these DNA boxes. Using *lacZ* transcriptional fusions of truncated P_{yirB} (as shown in Fig 3.6H), we found this to be the case: deletion of YuxN in WT led to a dramatic increase in beta-galactosidase activity when the promoter contained both DNA boxes (i.e. see promoter *i*), however this deletion had virtually no effect on the *yirB* promoters lacking one or both palindromes. Thus, we conclude that the palindromes are indeed YuxN boxes and that these two sites function cooperatively (Fig 3.6H). Next, we studied whether YuxN regulated the expression of *yirB* and/or *yuxN* itself. Deletion of YuxN resulted in a dramatic increase in the basal levels of both *yirB* and *yuxN* expression, and ectopic complementation restored the WT phenotype (Fig 3.6E). These results are consistent with YuxN acting as a repressor of both *yirB* and *yuxN*. If CssR functions as an anti-repressor for YuxN, then deletion of *cssR* should have no effect in a strain lacking YuxN. Indeed, this is the case since P_{yirB} is both fully derepressed and unresponsive to vancomycin in the Δ*cssR* Δ*yuxN* strain, as seen for the Δ*yuxN* mutant (Fig 3.6F). The cooperative role of the two YuxN boxes in mediating repression of *yirB* (Fig 3.6H) leads us to speculate that YuxN may form a repression loop that prevents access of RNA polymerase to both the *yirB* and *yuxN* promoters. Binding of CssR~P to CssR BoxI likely prevents YuxN binding to the overlapping binding site, alleviates YuxN repression and allows transcription. This model also explains the induction of *yuxN* in response to cell wall stress (Fig 3.6G), even though no apparent CssR binding sites are located upstream of P_{yuxN} (Fig S4).

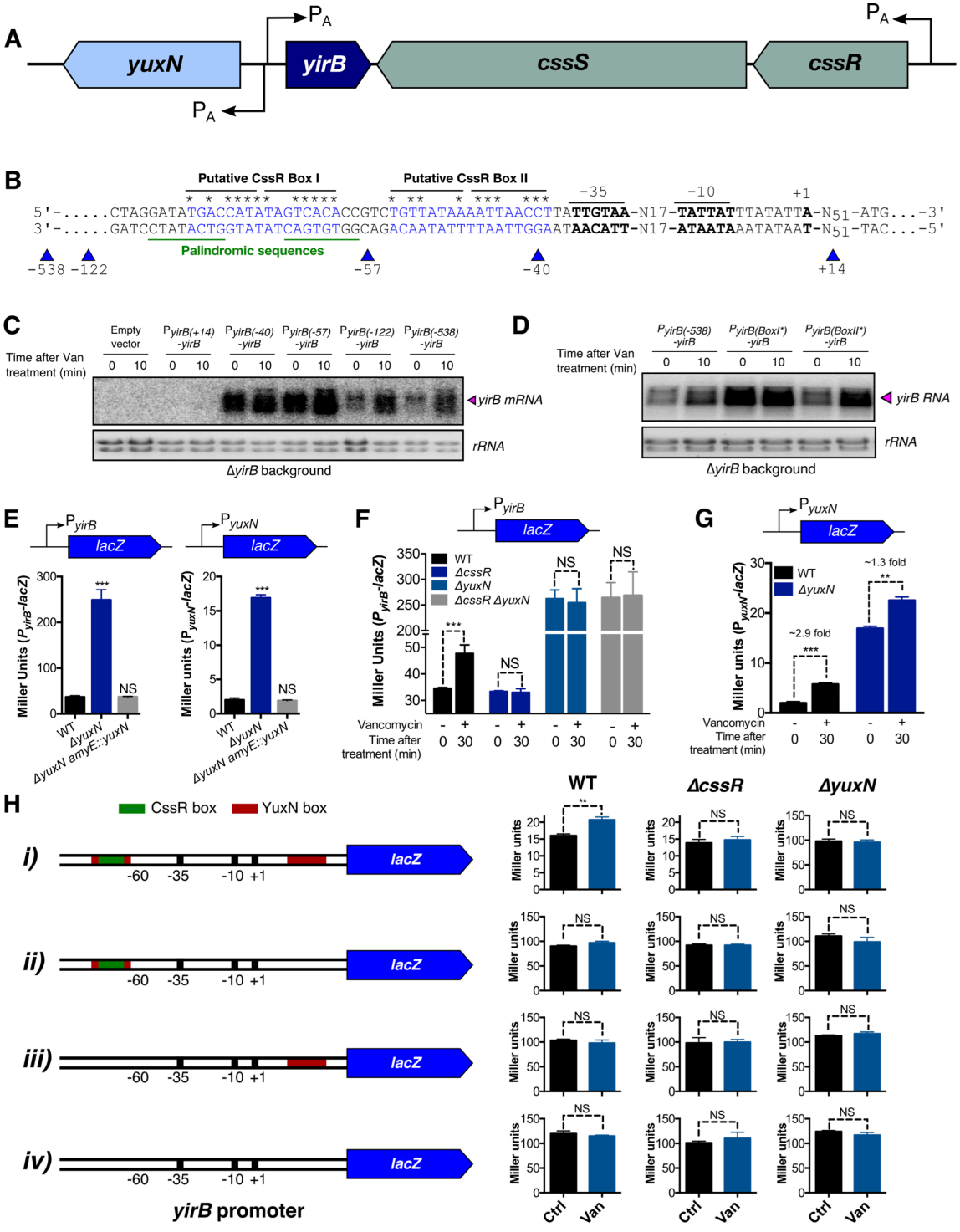

FIG 3.6 CSSR~P INDUCES YIRB EXPRESSION BY ANTAGONIZING YUXN REPRESSION

A) Genetic context of the *yirB* gene. B) The transcription start site was mapped using 5' RACE, and the identification of the putative -10 and -35 boxes was performed manually. Two putative CssR boxes were located upstream the -35 box, which exhibited similarity with the consensus CssR binding sequences (Fig S3). C) Promoter truncation analysis was used to

determine the contribution of the upstream DNA sequences on *yirB* regulation. For this, the *yirB* gene along with the promoter truncations were integrated at the *amyE* site and used to complement a Δ*yirB* mutant. The positions were mapped with respect to the +1 site as shown in Fig 3.6B. The *yirB* mRNA levels were determined by northern blot. D) The mRNA *yirB* levels were studied by northern blot in cells harboring the mutant CssR BoxI* (5'-TGACttTtTAGatAtt-3') or CssR BoxII* (5'-aGaaATAAAATTAAaC-3'), and compared with $P_{yirB(-538)}$-*yirB*. The three *yirB* promoter regions are identical except for the point mutations. The lower-case letters indicate the sites were the mutations were introduced (see Fig 3.6B). E) Analysis of the P_{yirB}-*lacZ* and P_{yuxN}-*lacZ* reporter fusions in cells lacking YuxN. F) Activity of the *yirB* promoter in WT, as well as in the *ΔcssR*, *ΔyuxN*, and *ΔcssR ΔyuxN* knockout mutants. G) The *yuxN* gene is upregulated in response to cell wall stress. (H) Analysis of the *yirB* promoter featuring truncations in the YuxN boxes. The different promoters were fused to the *lacZ* gene and its activity measured in WT, *ΔcssR*, and *ΔyuxN* cells before and after 20 min of treatment with 1 μg ml^{-1} vancomycin. Error bars represent SEM of at least three independent replicates. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, as estimated using one-way ANOVA and the Tukey's HSD test for Figure 3.6E, and the T-test for figures 3.6F-H. NS indicates no significant differences.

3.4 Discussion

The accumulation of Spx and the induction of its regulon in response to disulfide stress occurs through reduced proteolysis (Larsson *et al.*, 2007; Zhang and Zuber, 2007; Garg *et al.*, 2009; Engman and Wachenfeldt, 2015). Proteolysis is regulated by 1) oxidation and aggregation of the adaptor protein YjbH (Garg *et al.*, 2009; Engman and Wachenfeldt, 2015) and 2) decrease in proteolytic activity of the ClpXP protease (Zhang and Zuber, 2007). In response to cell wall stress, and unlike disulfide stress, transcriptional induction of *spx* takes place and is required for maximal accumulation of Spx and induction of the regulon (Rojas-Tapias and Helmann, 2018). Once the Spx protein is accumulated it primarily remains in the reduced state; reduced Spx is then capable of modulating transcription (Rojas-Tapias and Helmann, 2018). Notably, we observed that although transcriptional induction is critical for cell wall stress induction of the Spx regulon, a post-transcriptional event was also implicated in this response (Fig 3.1) (Rojas-Tapias and Helmann, 2018). Here, we report that, in addition to transcriptional control (Rojas-Tapias and Helmann, 2018), Spx stabilization against ClpXP-mediated proteolysis is also required for full and timely induction of Spx-controlled genes in response to cell wall stress. Remarkably, we found that, unlike disulfide stress, this stabilization during cell wall stress is mediated by the anti-adaptor protein YirB.

YirB was originally identified, through a yeast two-hybrid screen for YjbH-interacting proteins, as a small basic protein that was able to modulate Spx protein levels when artificially overexpressed (Kommineni *et al.*, 2011). YirB was found to modulate Spx levels through direct binding to the adaptor protein YjbH, which resulted in reduced binding of YjbH with Spx and therefore reduced ClpXP-mediated Spx proteolysis (Kommineni *et al.*, 2011). Although YirB bound YjbH with high affinity, and its overexpression significantly increased the stability of Spx, YirB did not affect Spx accumulation in response to diamide treatment. This suggested that YirB was likely important under other stress conditions. Cell wall stress indeed provides such a condition, as the regulatory mechanisms that result in induction of the Spx regulon in response to cell wall antibiotics display remarkable differences relative to disulfide stress (Rojas-Tapias and Helmann, 2018). Analysis of cells with conditional or native control of *spx* indeed showed that cells lacking YirB display reduced accumulation of Spx under both cell wall stress and active growth (Fig 3.2, 3.3, and 3.4).

The *yirB* gene lies upstream of the *cssRS* two-component system, and divergent from a gene encoding a putative transcription factor YuxN, a repressor protein of the TetR family. The genetic proximity between *yirB* and *cssRS*, as well as the correlation in the expression database between *htrB*, a CssRS-controlled gene, and *yirB* rendered the CssRS TCS as an attractive candidate for regulation of *yirB* under cell wall stress (Nicolas *et al.*, 2012). Genetic and transcriptomic analyses of the expression of *yirB* revealed that CssRS is indeed required for the transcriptional induction of *yirB* under cell wall stress (Fig 3.5). Remarkably, we found that YuxN represses *yirB*, and CssR~P appears to be required as an anti-repressor to antagonize YuxN (Fig 3.6). In agreement with previous findings (Kommineni *et al.*, 2011), diamide treatment did not lead to induction of the *yirB* gene, nor did deletion of *yirB* have a significant impact on the induction of *trxB* in the presence of diamide (Fig 3.4), suggesting that the stabilization of Spx mediated by YirB represents a hallmark of cell wall stress.

The CssRS TCS has shown to be induced under hypersecretion of soluble proteins such as the α-amylase, and therefore has been long associated to protein secretion stress (Hyyryläinen *et al.*,

2001; Noone *et al.*, 2012). The specific molecular signals that lead to its induction, however, are not yet fully understood (Noone *et al.*, 2012). Interestingly, cell wall stress also led to induction of CssRS, as upregulation of *htrB* and *yirB* took place following vancomycin treatment. Previous transcriptomic studies also revealed induction of *htrB* in response to cell wall stress (Cao *et al.*, 2002; Wecke *et al.*, 2011). We hypothesize that two events might potentially result in induction of CssRS under cell wall stress. First, the induction of regulons such as σ^M, σ^W, or LiaRS (which include several lipoproteins and membrane proteins) might lead to secretion stress. Indeed, mutants lacking σ^W displayed reduced induction of the CssRS regulon, however mutants lacking σ^M or LiaR exhibited increased CssRS activity (Fig S6). Alternatively, protein aggregation might occur as a direct effect of cell wall damage under antibiotic treatment. Further studies are required to unveil the underlying mechanisms. Induction of Spx may be advantageous under secretion stress since Spx controls the expression of protein chaperones and proteases (Rochat *et al.*, 2012).

The induction of the Spx regulon in response to cell wall stress in *B. subtilis* thus involves the timely expression of *spx* itself by σ^M (an ECF sigma factor) and the anti-adaptor *yirB* by CssRS (a two-component system) (Fig 3.7). YirB is more important for early induction of the regulon, while upregulation of *spx* appears to be more important in later stages (Fig 3.7). Although the role of Spx in adaptation to cell wall antibiotics remains undefined, this study provides further evidence of the regulation mechanisms that control its induction. Importantly, the regulation mechanisms that govern the induction of the Spx regulon in response to cell wall stress and disulfide stress take place through largely independent pathways, and thus provide a notable example of orthogonality in signal transduction systems. Our findings suggest a critical role of YirB in the activation of the Spx regulon; however, accumulation of Spx still occurs in cells lacking YirB (Fig 3.3 and 3.4), suggesting that further mechanisms are at play.

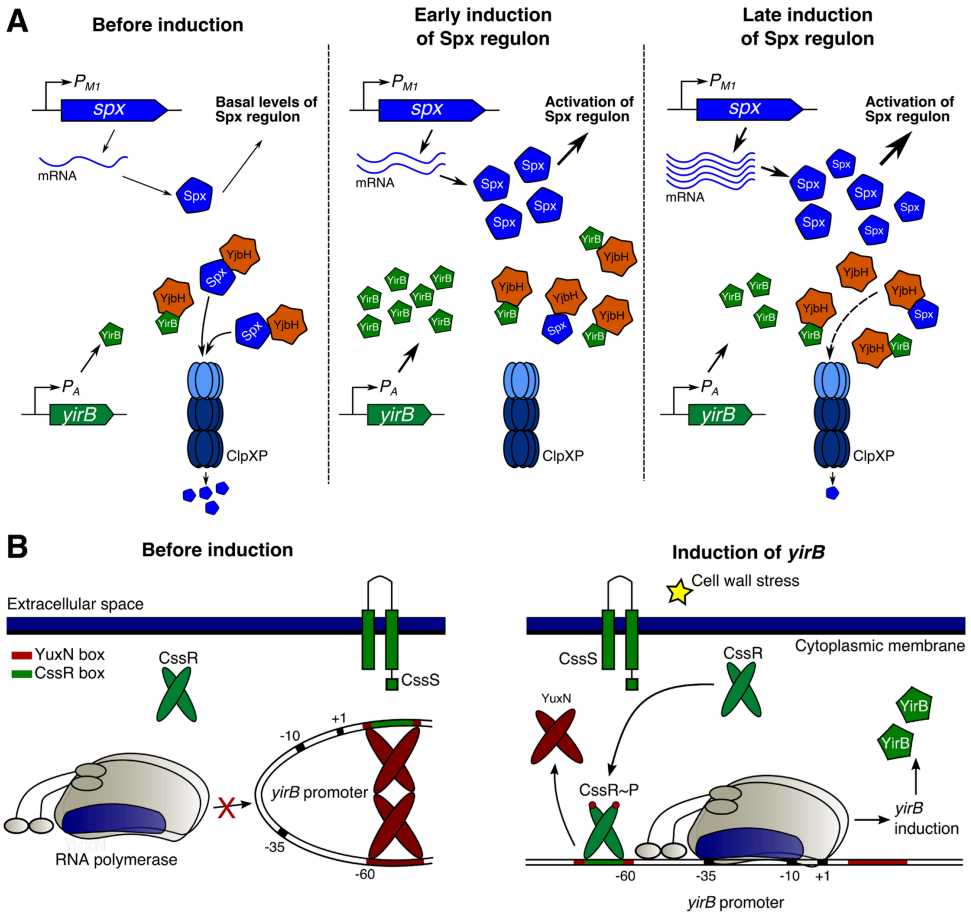

FIG 3.7. MODEL OF REGULATION OF SPX AND YIRB UNDER CELL WALL STRESS

A) Model of induction of the Spx regulon under cell wall stress. In exponentially growing cells (before induction), *spx* is constitutively expressed but Spx is actively degraded by the coordinated action of YjbH (i.e. the adaptor protein) and ClpXP (i.e. the protease). A fraction of Spx, however, remains stable at least in part due to the activity of YirB. This fraction of Spx, which is partially oxidized [4], drives the expression of Spx-controlled genes. After treatment with cell wall antibiotics (i.e. early induction), σ^M activates the expression of *spx* through the P_{M1} promoter, and CssR~P activates transcription of *yirB*. YirB then plays an important role in the stabilization of the newly synthesized Spx. Later in the process (i.e. late induction), induction of *spx* through P_{M1} is maximal, which leads to an increase in the amount of newly synthesized Spx. At the same time, the expression of *yirB* and presumably its role in stabilization decreases. The accumulation of Spx leads to induction of the genes in its regulon. B) Model for regulation of *yirB.* The protein YuxN (here presented as a dimer for simplicity) binds the YuxN boxes upstream and downstream the *yirB* promoter forming a DNA loop. Upon treatment with cell wall antibiotics, the CssR protein becomes phosphorylated by the CssS histidine kinase. CssR~P then binds the CssR BoxI, which overlaps the YuxN box, leading to derepression of the *yirB* promoter. The synthesized YirB protein binds YjbH and prevents Spx proteolysis.

3.5 Experimental procedures

3.5.1 Bacterial strains and culture conditions

All bacterial strains are listed in Table S1. *Bacillus subtilis* strains (all based on the *B. subtilis* 168 wild-type) were grown under standard conditions: lysogeny broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) broth at 37 ºC with vigorous shaking, unless otherwise stated. *Escherichia coli* DH5α was used for plasmid construction. Antibiotics were added to the growth medium when appropriate: 100 μg ml^{-1} ampicillin for *E. coli*, and 1 μg ml^{-1} erythromycin plus 25 μg ml^{-1} of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg ml^{-1} chloramphenicol, 100 μg ml^{-1} spectinomycin, and 10 μg ml^{-1} kanamycin for *B. subtilis*.

3.5.2 Strains construction

The knockout mutants were obtained from BGSC (Bacillus Genomic Stock Center), and the erythromycin cassette removed using the plasmid pDR244 (Koo *et al.*, 2017). The detailed protocol for construction of the strains is described in the Supplemental Experimental Procedures. The list of strains and primers used in this study are listed in Table S1 and Table S2.

3.5.3 Western blot

A total of 5 ml of cells were collected, washed in PBS, and resuspended in 150 μl of disruption buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% glycerol) supplemented with the cOmplete™ EDTA-free Protease Inhibitor Cocktail. The cells were disrupted by sonication, and then centrifuged for 15 min at 13,500 rpm at 4ºC. The soluble fraction was collected and quantified using the Bradford Assay. Reducing sample buffer was added to the protein extract, and then 5 μg of protein were loaded in a 4-20% SDS-PAGE. Proteins were transfer onto a PVDF membrane using the

TransBlot Turbo Transfer System (Bio-Rad, USA). The membrane was blocked using 5% protein blotting blocker dissolved in TTBS for 1 h. at RT. Then, the primary antibodies were resuspended in 0.5% protein blotting blocker dissolved in TTBS and incubated for 16 h at 4ºC. Finally, an anti-rabbit HRP-conjugated secondary antibody was added and incubated for 2 h at RT. The membrane was revealed using the Clarity Western ECL substrate and visualized in a Gel documenter. Protein fractionation was performed as previously described (Engman and Wachenfeldt, 2015). For quantification of Spx, the intensity of the bands was measured using the Image Lab 5.2.1 software (Bio-Rad, USA)

3.5.4 β-galactosidase activity

The cells were grown until OD_{600} reached ~0.5. Then, cells were treated or not with different chemicals, and incubated at 37ºC with agitation. After specific time points, samples were taken, washed twice in PBS, and finally resuspended in 900 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, $MgSO_4 \cdot 7H_2O$) supplemented with 400 μM DTT. Optical density at 600 nm was measured, and then the cells were lysed using 100 μg ml^{-1} lysozyme at 37ºC for 30 min. Next, 200 μl of 4 mg ml^{-1} ONPG were added to the lysate, and the reaction was incubated at 28ºC until the samples produced a visible yellow color. The reaction was stopped by adding 500 μl of 1.0 M Na_2CO_3. The absorbance was then measured at 420 nm and 550 nm, and β-galactosidase activity was determined using the following equation: Miller Units = $1000*[OD_{420}-1.75*OD_{550}]/(t*v*OD_{600})$, where t is time in minutes and v is the volume of culture used in the reaction. It is important to note that the values of β-galactosidase activity after treatment with cell wall active antibiotics might underestimate the effect of the drug on gene expression. This result was previously noted using another stable protein reporter (i.e. GFP) and is due to partial lysis elicited by antibiotic treatment.

3.5.5 Northern blot

RNA was isolated using the hot phenol-chloroform method as previously described (Rojas-Tapias and Helmann, 2018). RNA concentration and purity were determined using spectrophotometry, while RNA integrity was checked using denaturing agarose gels. Northern blot was performed on nylon membranes using radiolabeled RNA probes as previously described (Rojas-Tapias and Helmann, 2018). The *yirB* RNA probe was obtained using the primers P45 and DR282; the *htrB* probe was generated using the primers P47 and DR283; and the *spx* probe was obtained using the primers DR319 and DR320.

3.5.6 RT-qPCR

The RT-qPCR was performed as previously reported (Rojas-Tapias and Helmann, 2018). The primers used for *spx* were P11 and P12, for *sigM* were P31 and P32, for *trxB* were P13 and P14, for *yjbH* were P17 and P18, and for *gyrA* were P33 and P34.

3.5.7 Protein chase experiments

In order to determine the stability of Spx in WT and Δ*yirB*, cells were grown on 50 ml of LB broth up to OD_{600} = 0.5. Then vancomycin was added to a final concentration of 1 μg ml^{-1} to induce the stress response, cells were incubated for 10 min at 37ºC with shaking, and then pre-warmed chloramphenicol [100 μg ml^{-1}, final concentration] was added to stop protein synthesis. Samples (1.5 ml) were taken after 0, 1, 2, 3, and 5 min, and proteolysis was stopped by mixing the cells with 150 μl of pre-chilled 100% trichloroacetic acid (TCA). Cell suspensions were centrifuged at 13,500 rpm for 10 min at 4ºC, and the pellet was washed twice with ice-cold acetone in order to remove all TCA. The cell pellets were air dried for 10 min, resuspended in 130 μL of solubilization buffer (1% SDS, 1 mM

EDTA, 100 mM Tris-HCl, pH 8.0), and sonicated. A volume of 5 μl of the protein sample was load in a 4-20% SDS-PAGE gel, and western blot was carried out as previously described.

3.5.8 Mapping of the start transcription site by 5' RACE

Cells were grown up to OD_{600} = 0.5 and treated with 1 μg ml^{-1} vancomycin. After 10 min of incubation, 5 ml of sample were collected, and RNA was isolated using the RNeasy Kit (Qiagen) following manufacturer's instructions. The RNA was treated with Turbo DNase, and then purified by phenol-chloroform extraction. The RNA was quantified using a Nanodrop, its purity assessed by the 260/280 ratio, and integrity monitored by agarose gel electrophoresis. A total of 1 μg of RNA was reverse transcribed using the P46 primer, and the Reverse Transcription Reagents (Thermo Fisher Scientific, US) following manufacturer's instructions. The cDNA was column purified and then treated with the terminal transferase enzyme using CTP to add a homopolymeric cytosine tail at the 3' end. Then, the cDNA was PCR amplified using the AAP and DR288 primers by using a touchdown PCR followed by a conventional PCR. The PCR product was verified by electrophoresis and sequenced using the DR289 primer.

3.6 Acknowledgments

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3.7 References

Antelmann, H., Scharf, C., Hecker, M., and Hecker, M. (2000) Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis. *J Bacteriol* 182: 4478–4490.

Battesti, A., and Gottesman, S. (2013) Roles of adaptor proteins in regulation of bacterial proteolysis. *Curr Opin Microbiol* 16: 140–147.

Bougdour, A., Cunning, C., Baptiste, P.J., Elliott, T., and Gottesman, S. (2008) Multiple pathways for regulation of σS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. *Mol Microbiol* 68: 298–313.

Bougdour, A., Wickner, S., and Gottesman, S. (2006) Modulating RssB activity: IraP, a novel regulator of sigma(S) stability in *Escherichia coli*. *Genes Dev* 20: 884–897.

Browning, D.F., and Busby, S.J.W. (2016) Local and global regulation of transcription initiation in bacteria. *Nat Rev Microbiol* 14: 638–650.

Cao, M., Wang, T., Ye, R., and Helmann, J.D. (2002) Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ^W and σ^M regulons. *Mol Microbiol* 45: 1267–1276.

Chan, C.M., Hahn, E., and Zuber, P. (2014) Adaptor bypass mutations of *Bacillus subtilis spx* suggest a mechanism for YjbH-enhanced proteolysis of the regulator Spx by ClpXP. *Mol Microbiol* 93: 426–438.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O.P., Devine, K.M., and Dijl, J.M.V. (2002) A Novel Class of Heat and Secretion Stress-Responsive Genes Is Controlled by the Autoregulated CssRS Two-Component System of *Bacillus subtilis*. *J Bacteriol* 184: 5661–5671.

Eiamphungporn, W., and Helmann, J.D. (2008) The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. *Mol Microbiol* 67: 830–848.

Engman, J., and Wachenfeldt, von, C. (2015) Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. *Mol Microbiol* 95: 51–63.

Gaballa, A., Antelmann, H., Hamilton, C.J., and Helmann, J.D. (2013) Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx. *Microbiology* 159: 2025–2035.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009) The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *J Bacteriol* 191: 1268–1277.

Helmann, J.D. (2016) *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Curr Opin Microbiol* 30: 122–132.

Hyyryläinen, H.L., Bolhuis, A., Darmon, E., Muukkonen, L., Koski, P., Vitikainen, M., *et al.* (2001) A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. *Mol Microbiol* 41: 1159–1172.

Jordan, S., Hutchings, M.I., and Mascher, T. (2008) Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol Rev* 32: 107–146.

Kirstein, J., MoliEre, N., Dougan, D.A., and Turgay, K. (2009) Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat Rev Microbiol* 7: 589–599.

Kommineni, S., Garg, S.K., Chan, C.M., and Zuber, P. (2011) YjbH-enhanced proteolysis of Spx by ClpXP in *Bacillus subtilis* is inhibited by the small protein YirB (YuzO). *J Bacteriol* 193: 2133–2140.

Koo, B.-M., Kritikos, G., Farelli, J.D., Todor, H., Tong, K., Kimsey, H., *et al.* (2017) Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus subtilis*. *Cell Syst* 4: 291–305.e7.

Larsson, J.T., Rogstam, A., and Wachenfeldt, von, C. (2007) YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. *Mol Microbiol* 66: 669–684.

Leelakriangsak, M., and Zuber, P. (2007) Transcription from the P_3 promoter of the *Bacillus subtilis spx* gene is induced in response to disulfide stress. *J Bacteriol* 189: 1727–1735.

Leelakriangsak, M., Kobayashi, K., and Zuber, P. (2007) Dual negative control of spx transcription initiation from the P_3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *J Bacteriol* 189: 1736–1744.

Nakano, S., Erwin, K.N., Ralle, M., and Zuber, P. (2005) Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol* 55: 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A.D., and Zuber, P. (2003) Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in Bacillus subtilis. *Proc Natl Acad Sci USA* 100: 13603–13608.

Nakano, S., Zheng, G., Nakano, M.M., and Zuber, P. (2002) Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. *J Bacteriol* 184: 3664–3670.

Nicolas, P., Nicolas, P., Mader, U., Mäder, U., Dervyn, E., Dervyn, E., *et al.* (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science (New York, NY)* 335: 1103–1106.

Noone, D., Botella, E., Butler, C., Hansen, A., Jende, I., and Devine, K.M. (2012) Signal Perception by the Secretion Stress-Responsive CssRS Two-Component System in Bacillus subtilis. *J Bacteriol* 194: 1800–1814.

Pamp, S.J., Frees, D., Engelmann, S., Hecker, M., and Ingmer, H. (2006) Spx Is a Global Effector Impacting Stress Tolerance and Biofilm Formation in *Staphylococcus aureus*. *J Bacteriol* 188: 4861–4870.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinova, A., Korelusova, J., Leduc, A., *et al.* (2012) Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. *Nucleic Acids Res* 40: 9571–9583.

Rojas-Tapias, D.F., and Helmann, J.D. (2018) Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*. *Mol Microbiol* 107: 659–674.

Runde, S., MoliEre, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., *et al.* (2014) The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *Bacillus subtilis*. *Mol Microbiol* 91: 1036–1052.

Turlan, C., Prudhomme, M., Fichant, G., Martin, B., and Gutierrez, C. (2009) SpxA1, a novel transcriptional regulator involved in X-state (competence) development in *Streptococcus pneumoniae*. *Mol Microbiol* 73: 492–506.

Veiga, P., Bulbarela-Sampieri, C., Furlan, S., Maisons, A., Chapot-Chartier, M.-P., Erkelenz, M., *et al.* (2007) SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. *J Biol Chem* 282: 19342–19354.

Wecke, T., Bauer, T., Harth, H., Mäder, U., and Mascher, T. (2011) The rhamnolipid stress response of *Bacillus subtilis*. *FEMS Microbiol Lett* 323: 113–123.

Zhang, Y., and Zuber, P. (2007) Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. *J Bacteriol* 189: 7669–7680.

3.8 Supplementary information

3.8.1 Supplementary Figures

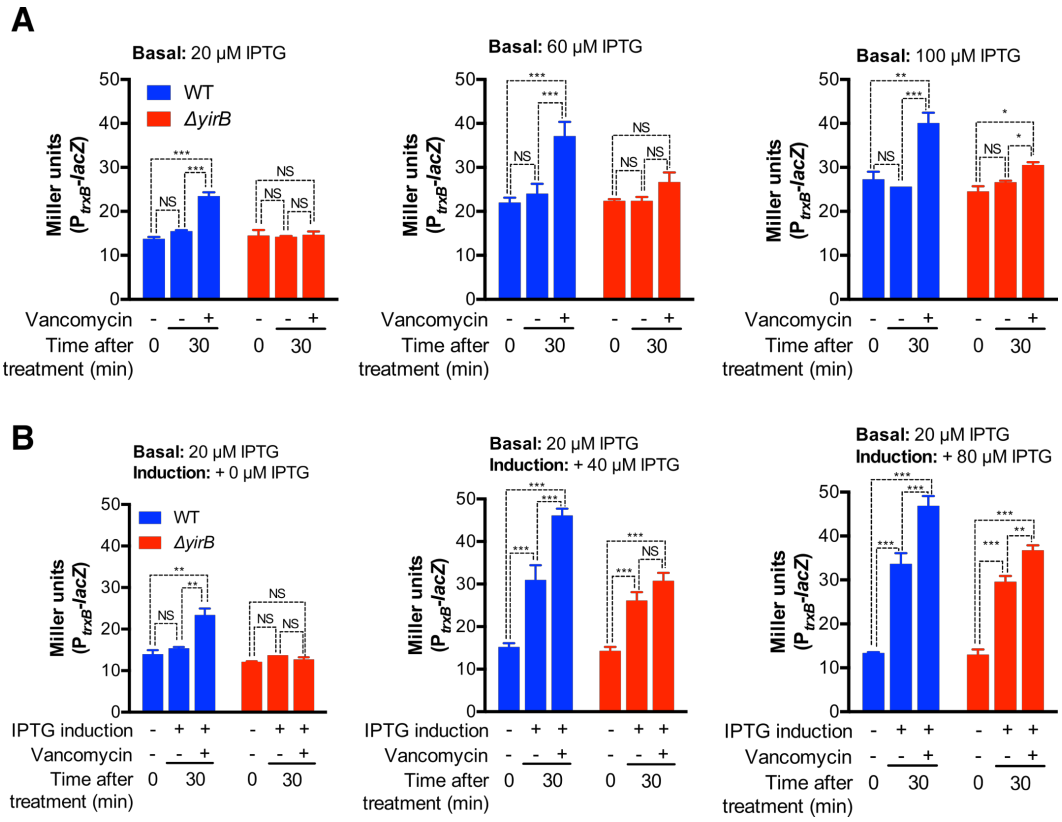

Fig S1 YirB also affects *trxB* induction in cells with conditional expression of *spx*. The induction of the Spx-dependent gene *trxB* was monitored 0 min. and 30 min. after treatment using a P_{trxB}-*lacZ* transcriptional fusion integrated at the *thrC* locus. A) The *spx* gene was constitutively expressed by addition of various concentrations of IPTG (i.e. 20 μM, 60 μM, and 100 μM), and then the cells were treated or not with 1 μg ml^{-1}vancomycin. B) The expression of *spx* was artificially induced. For this, cells were grown in the presence of 20 μM IPTG, and induction of the gene was achieved by using IPTG to reach 20 μM, 60 μM and 100 μM IPTG. Cells were treated or not with 1 μg ml^{-1} vancomycin. Error bars represent SEM of at least three independent replicates. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, as estimated using one-way ANOVA and the Tukey's HSD test. NS indicates no significant differences.

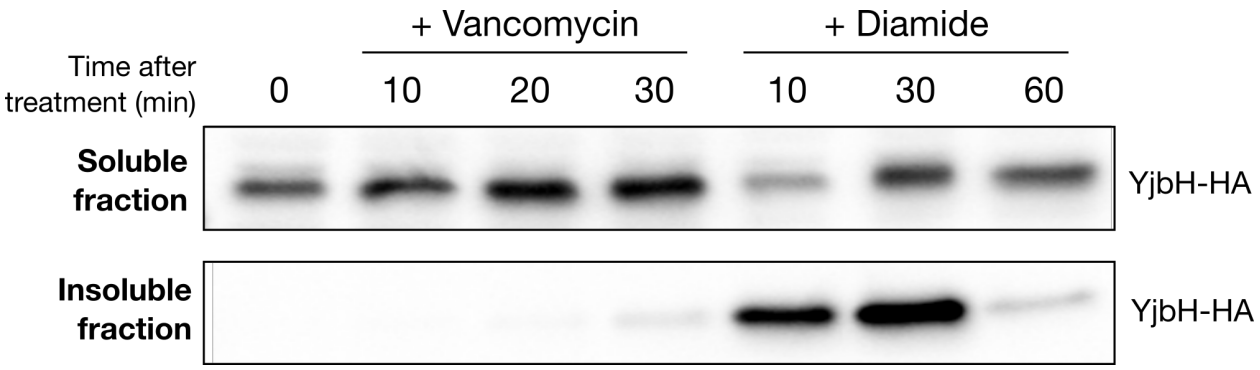

Fig S2 YjbH aggregation in response to vancomycin and diamide treatment.

YjbH-HA was studied in the soluble and insoluble protein fractions after treatment with 1 μg ml^{-1} vancomycin and 500 μM diamide. As observed, only diamide led to a significant accumulation of YjbH-HA in the insoluble fraction.

A

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)


htrB            TTTTCATTTTTATCCCA
htrA2           TTTTCATTTTTATCCCA
cssR            TTTTCATAATT-TCACA
htrA            TTTTCACAATT-TCCCA
                ******   ** ** **
```

B

Consensus CssR binding site based on the alignment above. The CssR response regulator is predicted to bind as a dimer on sequences repeated in tandem.

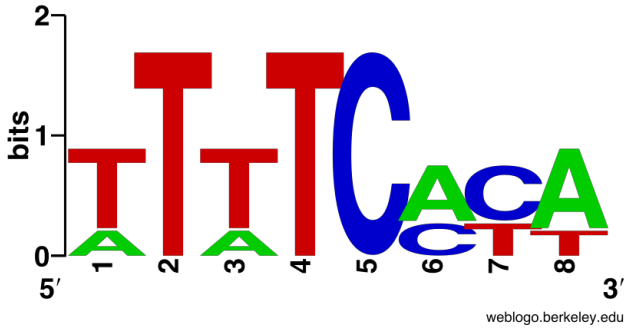

Fig S3 CssR binding sites. A) The known CssR boxes in the *Bacillus subtilis* 168 genome were aligned to determine the CssR box consensus sequence. B) A DNA logo was created for the consensus CssR box.

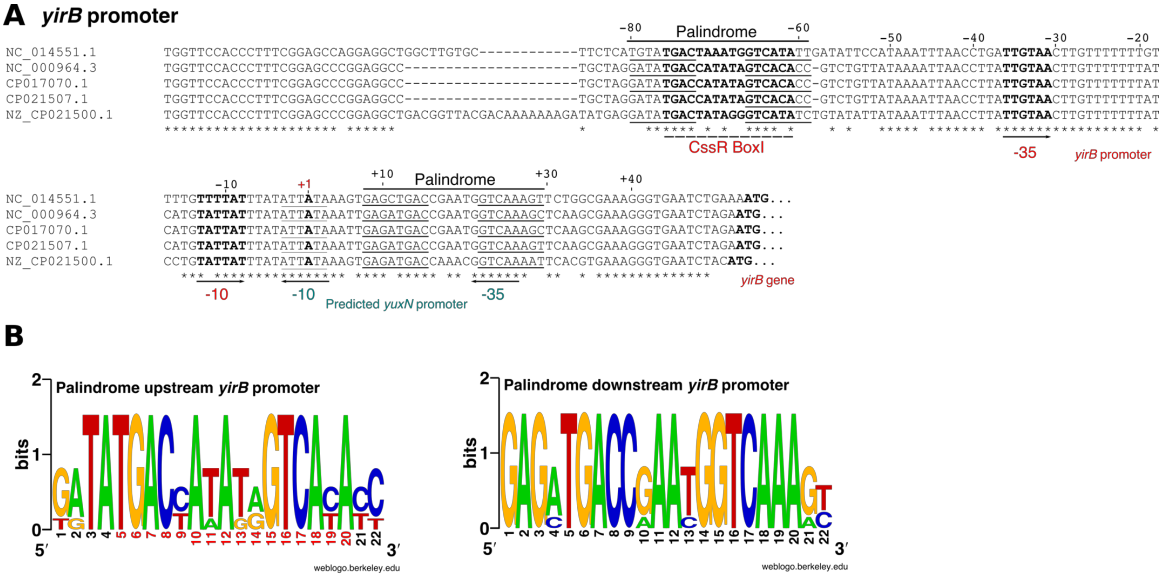

Fig S4 Palindromic sequences in the *yirB* and *yuxN* promoters. A) Promoter region of the *yirB* and *yuxN* promoters in different *Bacillus* species. In descending order, *Bacillus amyloliquefaciens* DSM7 [NC_014551.1], *Bacillus subtilis* strain 168 [NC_000964.3], *Bacillus gibsonii* strain FJAT-10019 [CP17070.1], *Bacillus atrophaeus* strain [SRCM101359], and *Bacillus licheniformis* strain [SRCM101441]. The mapped *yirB* promoter and the predicted *yuxN* promoter are also displayed, as well as the palindrome sequences found on both promoters. B) DNA logos were created for the *yirB* and *yuxN* palindromes, as observed they display remarkable similarity.

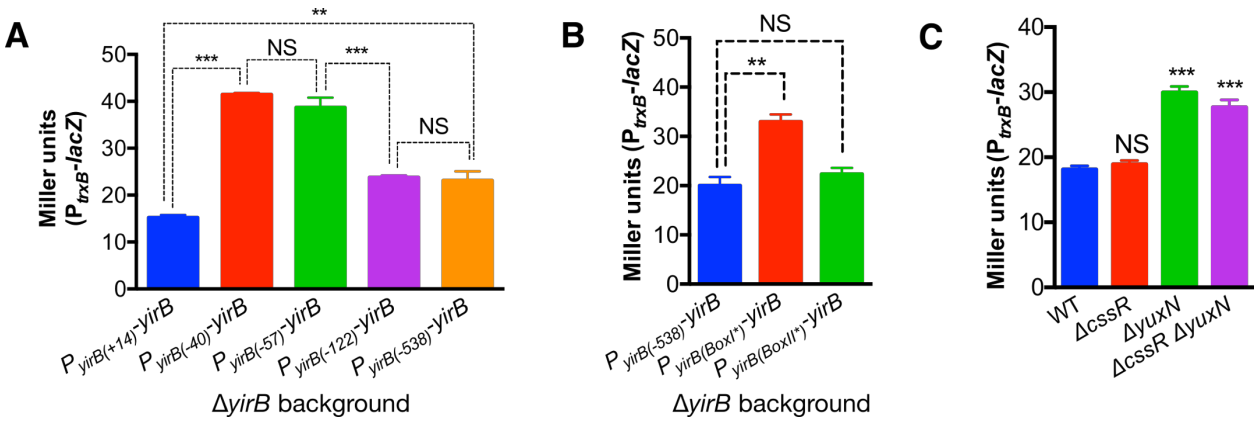

Fig S5 Effect of truncations, point mutations, and gene deletions on induction of the Spx-controlled gene *trxB*. A) Analysis of basal *trxB* activity in cells expressing the different *yirB* promoter truncations. Statistical analysis was performed in pairs using the T-test. B) Analysis of basal *trxB* activity in cells expressing the wild-type *yirB* promoter vs. the mutant *yirB* promoters harboring point mutations in the CssR BoxI boxes. Statistical analysis was performed using the Dunnett Test, comparing $P_{yirB(-538)}$ against promoters of the same length but including mutations in the CssR predicted boxes. C) Effect of the deletions of CssR, YuxN, and CssR & YuxN on basal expression levels of *trxB*. Statistical analysis was performed using the Dunnett Test comparing the mutant strains against WT. Error bars represent SEM of at least three independent replicates. One, two, and three asterisks indicate significant differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. NS indicates no significant differences.

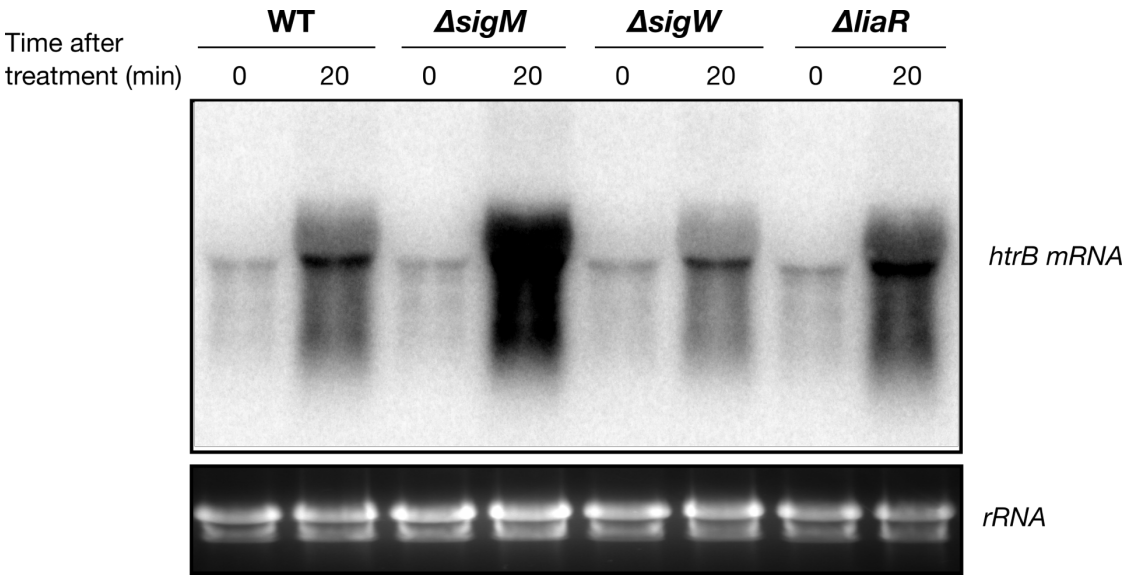

Fig S6 Effect of deletions of major transcription regulators involved in the cell wall stress response on activation of the CssRS regulon. Induction of the CssR regulon in response to vancomycin treatment in cells lacking SigM, SigW, or LiaR.

3.8.2 Supplementary Tables

Table S1. Strains used in this study.

Number	Genotype	Construction
HB18501	WT	Lab strain
HB18801	*spx::kan*	Lab strain
HB18504	*ΔyirB*	This study
HB18506	*ΔcssR*	This study
HB23044	*ΔyuxN*	This study
HB23078	*ΔyuxN ΔcssR*	This study
HB18905	*spx::$P_{spx(PM1)}$-yjbC-spx (kan)*	Lab strain
HB18903	*spx::$P_{spx(wt)}$-spx*	Lab strain
HB18658	*thrC*::P_{trxB}-*lacZ* (*ery*)	Lab strain
HB23089	*thrC::P_{trxA}-lacZ (ery)*	This study
HB18524	*thrC::P_{yirB}-lacZ (ery)*	This study
HB23091	*thrC::P_{yuxN}-lacZ (ery)*	This study
HB18805	*spx::kan amyE::P_{spac}-spx (spec)*	Lab strain
HB18595	*spx*::*kan amyE*::P_{spac}-*spx* (*spec*) *thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB18571	*ΔyirB spx*::*neo amyE*::P_{spac}-*spx* (*spec*) *thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB18510	*ΔyirB thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB18521	*ΔyirB amyE::$P_{yirB(-538, +51)}$- yirB (cm) thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB18554	*ΔcssR amyE::cssR (cm)*	This study
HB18564	*ΔcssR amyE::cssR^{D52A} (cm)*	This study
HB23030	*ΔyirB amyE::$P_{yirB(+14, +51)}$-yirB (cm) thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB23031	*ΔyirB amyE::$P_{yirB(-40, +51)}$-yirB (cm) thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB23032	*ΔyirB amyE::$P_{yirB(-57, +51)}$-yirB (cm) thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB23033	*ΔyirB amyE::$P_{yirB(-122, +51)}$-yirB (cm) thrC*::P_{trxB}-*lacZ* (*ery*)	This study
HB23016	*ΔyirB amyE::cm thrC*::P_{trxB}-*lacZ* (*ery*)	This study

HB23015	*ΔyirB amyE::$P_{yirB(BoxI)}$-yirB (cm) thrC::P_{trxB}-lacZ (ery)*	This study
HB18577	*ΔyirB amyE::$P_{yirB(BoxII)}$-yirB (cm) thrC::P_{trxB}-lacZ (ery)*	This study
HB18567	*ΔyirB thrC::P_{trxB}-lacZ (ery) spx::$P_{spx(wt)}$-spx (kan)*	This study
HB18568	*ΔyirB thrC::P_{trxB}-lacZ (ery) spx::$P_{spx(ΔPsigM1)}$-spx (kan)*	This study
HB18569	*thrC::P_{trxB}-lacZ (ery) spx::$P_{spx(wt)}$-spx (kan)*	Lab strain
HB18570	*thrC::P_{trxB}-lacZ (ery) spx::$P_{spx(ΔPsigM1)}$-spx (kan)*	Lab strain
HB18588	*yjbH-HA (spec)*	Lab strain
HB23008	*ΔyirB yjbH-HA (spec)*	This study
HB23132	*ΔyirB thrC::P_{trxA}-lacZ (ery)*	This study
HB23080	*ΔcssR thrC::P_{yirB}-lacZ (ery)*	This study
HB23082	*ΔyuxN thrC::P_{yirB}-lacZ (ery)*	This study
HB23084	*ΔyuxN ΔcssR thrC::P_{yirB}-lacZ (ery)*	This study
HB23079	*ΔcssR thrC::P_{trxB}-lacZ (ery)*	This study
HB23081	*ΔyuxN thrC::P_{trxB}-lacZ (ery)*	This study
HB23083	*ΔyuxN ΔcssR thrC::P_{trxB}-lacZ (ery)*	This study
HB23077	*ΔyuxN thrC::P_{yirB}-lacZ (ery) amyE::yuxN (cm)*	This study
HB23092	*ΔyuxN thrC::P_{yuxN}-lacZ (ery)*	This study
HB23136	*ΔyuxN amy::yuxN (cm) thrC::P_{yuxN}-lacZ (ery)*	This study
HB23133	*spx::$P_{spx(PM1)}$-yjbC-spx (kan) thrC::P_{trxA}-lacZ (ery)*	This study
HB23134	*ΔyirB spx:$P_{spx(PM1)}$-yjbC-spx (kan) thrC::P_{trxA}-lacZ (ery)*	This study
HB23171	*WT thrC::$P_{yirB(-128, +46)}$-lacZ (ery)*	This study
HB23172	*WT thrC::$P_{yirB(-128, +10)}$-lacZ (ery)*	This study
HB23173	*WT thrC::$P_{yirB(-57, +46)}$-lacZ (ery)*	This study
HB23174	*WT thrC::$P_{yirB(-57, +10)}$-lacZ (ery)*	This study
HB23175	*ΔcssR thrC::$P_{yirB(-128, +46)}$-lacZ (ery)*	This study

HB23176	*ΔcssR thrC::$P_{yirB(-128, +10)}$-lacZ (ery)*	This study
HB23177	*ΔcssR thrC::$P_{yirB(-57, +46)}$-lacZ (ery)*	This study
HB23178	*ΔcssR thrC::$P_{yirB(-57, +10)}$-lacZ (ery)*	This study
HB23179	*ΔyuxN thrC::$P_{yirB(-128, +46)}$-lacZ (ery)*	This study
HB23180	*ΔyuxN thrC::$P_{yirB(-128, +10)}$-lacZ (ery)*	This study
HB23181	*ΔyuxN thrC::$P_{yirB(-57, +46)}$-lacZ (ery)*	This study
HB23182	*ΔyuxN thrC::$P_{yirB(-57, +10)}$-lacZ (ery)*	This study
HB23183	*ΔcssR ΔyuxN thrC::$P_{yirB(-128, +46)}$-lacZ (ery)*	This study
HB23184	*ΔcssR ΔyuxN thrC::$P_{yirB(-128, +10)}$-lacZ (ery)*	This study
HB23185	*ΔcssR ΔyuxN thrC::$P_{yirB(-57, +46)}$-lacZ (ery)*	This study
HB23186	*ΔcssR ΔyuxN thrC::$P_{yirB(-57, +10)}$-lacZ (ery)*	This study

** The ¨Δ¨* symbol is used to indicate a markerless deletion generated using the pDR244 plasmid on a BKE strain of the Bacillus Genomic Stock Center.

Table S2. Primer sequences

Primer	*Sequence*
DR104	GCTTTTATATAGGGAAAAGGTGGTG
DR107	CCACCTTTTCCCTATATAAAAGCGGAATCTCCGCCGCCAACAAC
DR112	GGATCCCCAGCTTGTTGATACACGGACACGTAGGAATCTACTTAGG
DR113	GTGTATCAACAAGCTGGGGATCC
DR242	ATCAGAATTCCGCTTTTTTTCCATAGATGTTCAG
DR243	ATCAGGATCCCTCATTCTAGATTCACCCTTTCG
DR244	ATCGAAGCTTGACAAAACGGGTGTAACATACC
DR259	ATCGAAGCTTGGATCCTCATGATGACATCATCCTGTAGC
DR264	ATCGAGAATTCCTTACACTCCTTTAACGGTTATTC
DR278	GGATTCTCGCTATCATGCTGCCG
DR279	GCAGCATGATAGCGAGAATCCATAGGTGGGGAGACGGTGTC
DR282	ATCGATAATACGACTCACTATAGGTGTGAAAGAATAGCCGTTAAAATC
DR283	ATCGATAATACGACTCACTATAGGGAGAATATCCGCACTGCTTTCCAC
DR288	GCTGTTTGCATTGATGAACC
DR289	CATTTTTTCTCTGACAAGGTCTG
DR305	CCGTCAGAAATAAAATTAAACTTATTGTAACTTG
DR306	AGTTTAATTTTATTTCTGACGGTGTGACTATATGGTC
DR319	TGTACCGCTTGATCAACGAG
DR320	ATCGATAATACGACTCACTATAGGGAGAGCCAAACGCTGTGCTTCTC
DR340	GATATGACTTTTTAGATATTCCGTCTGTTATAAAATTAACCTTATTG
DR341	AATATCTAAAAAGTCATATCCTAGCAGGCCTCC
DR347	ATCAGAATTCGAATGGTCAAAGCTCAAGCG
DR348	ATCAGAATTCTTATTGTAACTTGTTTTTTTATC
DR349	ATCAGAATTCTCTGTTATAAAATTAACCTTATTGTAAC
DR350	ATCAGAATTCCCTTCAATGGTTCCACCCTTTC
DR387	ATCAGAATTCTTCACCCTTTCGCTTGAGCTTTG
DR388	ATCAGGATCCCTGACAGAGGAGCAATTTAACG

DR404	ATCAAAGCTTGTCAGTCGTCTTACTTACGCTG
DR405	ATCAGGATCCGATCAGTTGCTTTTACGATAGC
DR408	ATCAGGATCCCAGTTTTTCTTTTTCCTTCAATGG
DR430	ATCAGGATCCTCTCAATTTATAATATAAATAATACATG
DR431	ATCAGGATCCCCTTTCGCTTGAGCTTTGACC
P3	GCTCCTACACTTGGGAAGTCCAG
P4	GCAAGAGTTGCACTTCCGGG
P11	CCAAGCTGTACTTCATGCAGAAAGGC
P12	GATCAAGCGGTACAAATCTTGCAGC
P13	GCGTGCCTGGCGAAAAAGAATTGG
P14	GAGCACGGAGTTTATCACGTCTGTG
P17	GATGAAAACGTGCTTTTGGAGATTGCTG
P18	CTTTAAGCCCTTCATCCTCATGCTGC
P31	TGATCACAGCAAAGTAAAGCCCTGGC
P32	CAGGACTTTGAACAGCATTTTGAAAGAGG
P33	GCAACAAACATTCCTCCGCACCAG
P34	CTTTTGCCCGGATCGTGATAGAGC
P45	GAATTGGACGAAATGATCAGC
P46	TGTGAAAGAATAGCCGTTAAAATC
P47	TTTAGGCGTGCAGATGATTG
P48	ATATCCGCACTGCTTTCCAC

3.8.3 Supplementary Methods

3.8.3.1 *Strains construction*

The strains with clean deletions in the *yirB, cssR,* and *yuxN* genes were constructed by removing the erythromycin resistance cassette from the strains BKE33029, BKE33010, and BKE33030 using the pDR244 plasmid. The knockout mutant strains, the pDR244 plasmid, as well as the transformation method were obtained from the BGSC (Bacillus Genomic Stock Center). All the DNA primers used in this study are listed in Table S1.

The complementation of *yirB* was obtained by PCR amplification of the coding sequence and promoter region with the primers DR242 and DR244, which were cloned into the EcoR1 and HindIII restriction sites in pDG1662. The complementation of *cssR* was obtained by amplification with DR264 and DR259 into the same restriction sites into pDG1662. The complementation of *yuxN* was obtained by amplification with DR387 and DR388 and inserted into pDG1662.

The point mutant in the CssR phosphorylation site was performed using the mutagenic primers DR278 and DR279, which were used for overlap PCR along with the primers DR264 and DR259. The fragment containing the mutation was cloned into pDG1662.

The P_{trxB}-*lacZ* reporter was constructed by PCR amplification of the *trxB* promoter using the primers DR112 and DR107 and inserted into pDG1663 by Gibson assembly. The vector was amplified using DR104 and DR113, digested with DpnI, and used for Gibson cloning. The P_{trxA}-*lacZ,* P_{yirB}-*lacZ,* and P_{yuxN}-*lacZ* transcriptional fusions were constructed by PCR amplifying the promoter using the primers DR404 and DR405, DR242 and DR243, and DR387 and DR408, respectively. The fragments were cloned into the pDG1663 vector. All constructions were verified by PCR and sequencing. The reporters were transformed into competent *B. subtilis* 168 cells to make the different strains listed in Table S1.

For construction of the *yirB* promoter truncations the forward primers DR347, DR348, DR349, DR350, and DR242 with EcoRI restriction site, and the reverse primer DR244 with HindIII restriction site were used to amplify the different fragments. For mutagenesis of the predicted CssRS boxes the mutagenic primers DR305 and DR306, and DR340 and DR341 were used. The primers DR242 and DR244 were used as external primers. The inserts were cloned into pDG1662. All constructions were verified by PCR and sequencing.

For construction of the truncations in Fig 3.6H, the promoter regions were amplified using the primers: i) DR349 and DR431, ii) DR349 and DR430, iii) DR350 and DR431, and iv) DR350 and DR430. The fragments were then cloned into pDG1663 by restriction cloning. All constructions were verified by PCR and sequencing, and then transformed in HB18501, HB18506, HB23044, and HB23078 to produce the strains HB23171-HB23186.

CHAPTER IV: Regulation of Spx by the ClpCP protease in *Bacillus subtilis*

The present Chapter is in progress and on plans to be submitted as:

Rojas-Tapias, D.F., and Helmann, J.D. (2018) Regulation of *Bacillus subtilis* Spx by the ClpCP protease.

4.1 Summary

Spx is a transcription factor present in *Bacillus subtilis* and other Firmicutes. In *B. subtilis*, Spx drives the expression of a large regulon in response to several conditions including disulfide, heat, and cell wall stress. The regulatory mechanisms that lead to activation of the Spx regulon have proven to be remarkably complex, involving adjustments in transcription, proteolysis, and post-translational modifications. To identify new genes involved in Spx regulation, a transposon screen was performed to identify mutations affecting the activity of the *trxB* promoter (P_{trxB}), a reporter of Spx activity. Deletion of the master regulator of proteolysis CtsR, reduced the expression of P_{trxB}, which correlated with reduced Spx levels. This effect required ClpP, as WT and *ΔctsR* cells lacking this protease displayed similar P_{trxB} activity and Spx levels. Unexpectedly, this effect was not due to increased ClpXP proteolysis, but instead to ClpCP-mediated Spx degradation. Since ClpC requires adaptor proteins, we surveyed the role of the known adaptors on Spx proteolysis. Evidence suggests a major role of MecA in growing cells, as previously observed in *in vitro* experiments, and also a role for McsB in conditions that result in upregulation of *clpC*. We further identified a role for arginine phosphorylation in Spx regulation, as cells lacking the YwlE phosphatase displayed increased Spx proteolysis. Finally, we show that the *ctsR* operon is under simultaneous Spx and CtsR control. Altogether, this work provides evidence for a role of ClpCP in Spx regulation, and sheds light into new regulatory mechanisms associated with Spx activity/stability in *B. subtilis*.

4.2 Introduction

Spx belongs to the ArsC family of transcriptional regulators and is best known as the master regulator of the disulfide stress response in *B. subtilis* (Nakano et al., 2003). Orthologs of Spx are found in the Firmicutes, and their number varies among species (Chapter 1). The Spx protein consists of two major domains: one is formed by the N-terminal and C-terminal parts of the protein, and one formed by its central region. The first domain contains a CXXC redox-sensing switch that modulates the regulatory activity of Spx upon formation of an intramolecular disulfide bond. The central domain is involved in binding to the α-CTD domain of the RNA polymerase (Newberry *et al.*, 2005). Spx is an unusual transcription factor that affects transcription without direct DNA binding (Haugen et al., 2008; Lin et al., 2013).

The activity of Spx is necessary for the induction of some genes, for example *trxB,* which encodes the thioredoxin reductase (Nakano et al., 2005; Rojas-Tapias and Helmann, 2018a)*,* and is important for the activation and repression of many other genes (Rochat *et al.*, 2012). Spx controls the expression of a vast number of genes, including those involved in the synthesis of bacillithiol and cysteine, the thioredoxin system, and the HSP/Clp chaperones and proteases (Nakano et al., 2003; Rochat et al., 2012).

Spx is encoded in a bicistronic operon, along with a putative acetyltransferase, and its expression is regulated by at least three different promoters that are dependent on different holoforms of the RNA polymerase (Antelmann *et al.*, 2000; Leelakriangsak and Zuber, 2007; Rojas-Tapias and Helmann, 2018a). The P_A promoter, dependent on σ^A, has been shown to be sufficient to complement an *Δspx* mutant in response to disulfide stress (Leelakriangsak and Zuber, 2007). The activity of the P_A promoter is regulated by two protein repressors, PerR and YodB (Leelakriangsak *et al.*, 2007), and is therefore induced by redox or electrophile stress. The σ^M-controlled P_{M1} promoter was recently

shown to be critical for induction of the Spx regulon in response to cell wall stress response (Rojas-Tapias and Helmann, 2018a). Little is known, however, about the functional role of the P_B promoter, which is induced in response to phosphate starvation (Antelmann *et al.*, 2000).

Under unstressed conditions Spx levels remain low since the protein is actively proteolyzed via ClpXP and the adaptor protein YjbH (Garg et al., 2009; Larsson et al., 2007). Stabilization of Spx plays a critical role in the induction of the Spx regulon in response to disulfide stress as imposed by the electrophile diamide (Engman and Wachenfeldt, 2015; Rojas-Tapias and Helmann, 2018b). Under diamide stress, Spx is stabilized by aggregation of YjbH (Engman and Wachenfeldt, 2015) and a decrease in ClpXP activity (Zhang and Zuber, 2007). Induction by diamide also involves oxidation of the Spx redox-sensing switch (Engman and Wachenfeldt, 2015; Garg et al., 2009; Larsson et al., 2007; Zhang and Zuber, 2007), Previously, it was shown that another protease, ClpCP, is also capable of degrading Spx *in vitro*, however, the conditions in which this process occurs, if it does, remain unknown (Nakano et al., 2002b).

Although the *in vivo* mechanisms of Spx activation by stress are fairly well understood, some questions regarding Spx stability and activity remain to be answered. For instance, cells harboring a Spx^{C10A} or $Spx^{C10AC13A}$ protein, both unable to form the intramolecular disulfide switch, display a distinct behavior in response to cell wall and disulfide stress (Rojas-Tapias and Helmann, 2018a). Also, *B. subtilis* cells can accumulate Spx in response to cell wall stress in a YirB-independent manner (Rojas-Tapias and Helmann, 2018b). Finally, there is not always a direct correlation between Spx levels and the expression of Spx-controlled genes (unpublished data). These observations suggest that Spx activity is likely regulated through additional pathways.

Here, we identify additional regulators that affect Spx activity. We report that ClpCP also degrades Spx *in vivo*, this degradation depends on the MecA adaptor, and this is the dominant degradation pathway in cells after disulfide stress. Moreover, we provide evidence that changes in

arginine phosphorylation also affect Spx degradation, either directly or indirectly, and thereby affect induction of the Spx regulon.

4.3 Results

4.3.1 Transposon mutagenesis identifies CtsR as a potential modulator of Spx activity/stability

In order to identify novel pathways involved in Spx regulation, we carried out *mariner* transposon mutagenesis in both WT and Δ*yirB* cells. For this, we used cells harboring a P_{trxB}-*lacZ* fusion, which is positively regulated by Spx and serves as a readout of Spx activity. The transposon library was plated on LB + X-gal medium, and light blue or white colonies were selected for further analysis. Transposon-generated mutations that decreased Spx activity included *iolR* (8 independent insertions), *ctsR* (5) (Fig 4.1), *gndA* (1), *ywlE* (1), and *menH* (3). In this study, we focus on *ctsR* because CtsR is the master regulator of proteolysis (Derré et al., 1999; Kruger and Hecker, 1998), and hence a potential regulator of Spx stability. In addition, CtsR was reported to interact with YjbH in yeast two-hybrid experiments (Kommineni *et al.*, 2011), and the CtsR regulon is induced in response to disulfide stress (Elsholz et al., 2011; Rochat et al., 2012).

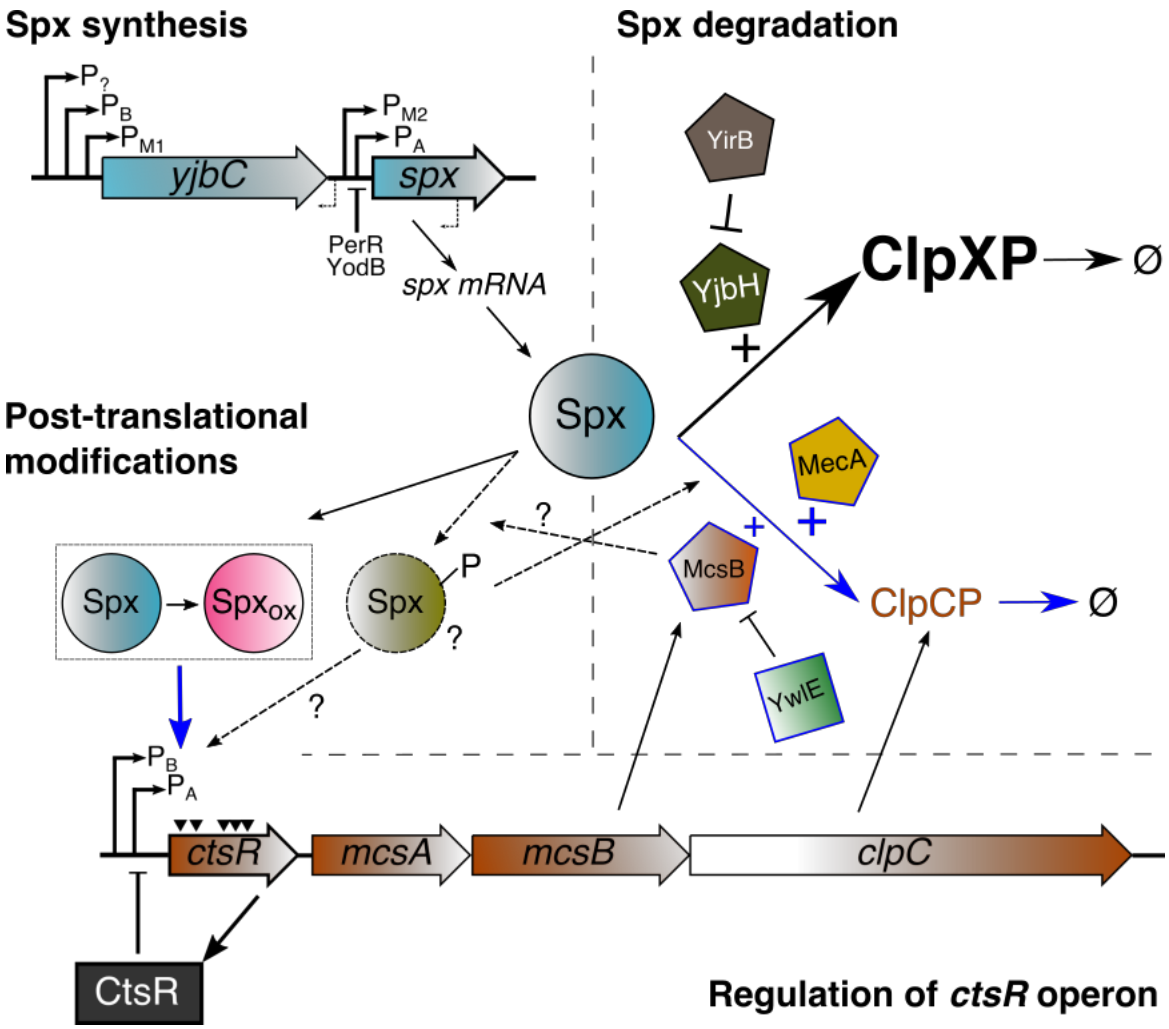

FIG 4.1 MODEL OF SPX REGULATION IN B. SUBTILIS.

A model of regulation of Spx in *B. subtilis* is presented. The model includes pathways for Spx synthesis and degradation, as well as post-translational modifications. Blue arrows indicate findings of this work, dotted arrows indicate aspects that are still unclear but inferred from this study, and black arrows represent aspects already known of *B. subtilis* regulation. The *ctsR* operon is also included in the model to illustrate the composition of the *ctsR* operon and its regulation, as well as the insertions obtained in the transposon screening. A total of five independent *mariner* insertions were found within the coding sequence of the *ctsR* gene, which encodes the master regulator of the energy-dependent Clp proteases in *B. subtilis* and other Firmicutes. The location of the transposon insertions are symbolized by black inverted triangles.

4.3.2 Dysregulation of the CtsR regulon leads to reduced induction of P_{trxB}

To validate the results obtained with the *ctsR*::mTn insertion, we constructed a strain harboring a clean deletion of the *ctsR* gene (*ΔctsR*), which also contained the P_{trxB}-*lacZ* reporter fusion. As expected, the *ΔctsR* null strain displayed whiter colonies compared to wild-type on LB plates supplemented with X-gal (Fig 4.2A). This result demonstrated that the observed low activity of P_{trxB} in the *ctsR*::mTn strain was due to inactivation of *ctsR* and not a polar effect on downstream genes. No changes in the growth rate were observed in cells lacking CtsR compared to WT (Fig 4.2B). Deletion of *ctsR* results in reduced expression of the P_{trxB}-*lacZ* fusion all along the growth curve (Fig 4.2C). As expected, ectopic complementation of the Δ*ctsR* null strain with a conditional allele of *ctsR (i.e.* P_{hs}-*ctsR)* restored the phenotype (Fig 4.2A, 4.2D). By contrast, complementation with the $ctsR^{R63E}$ allele, which encodes a CtsR protein unable to bind DNA (Fuhrmann et al., 2009), was unable to restore the wild-type phenotype (Fig 4.2A, 4.2D). Altogether, the present results indicate that the differences in P_{trxB} activity between both WT and Δ*ctsR* are likely the direct result of dysregulation of the CtsR regulon (Fig 4.2E).

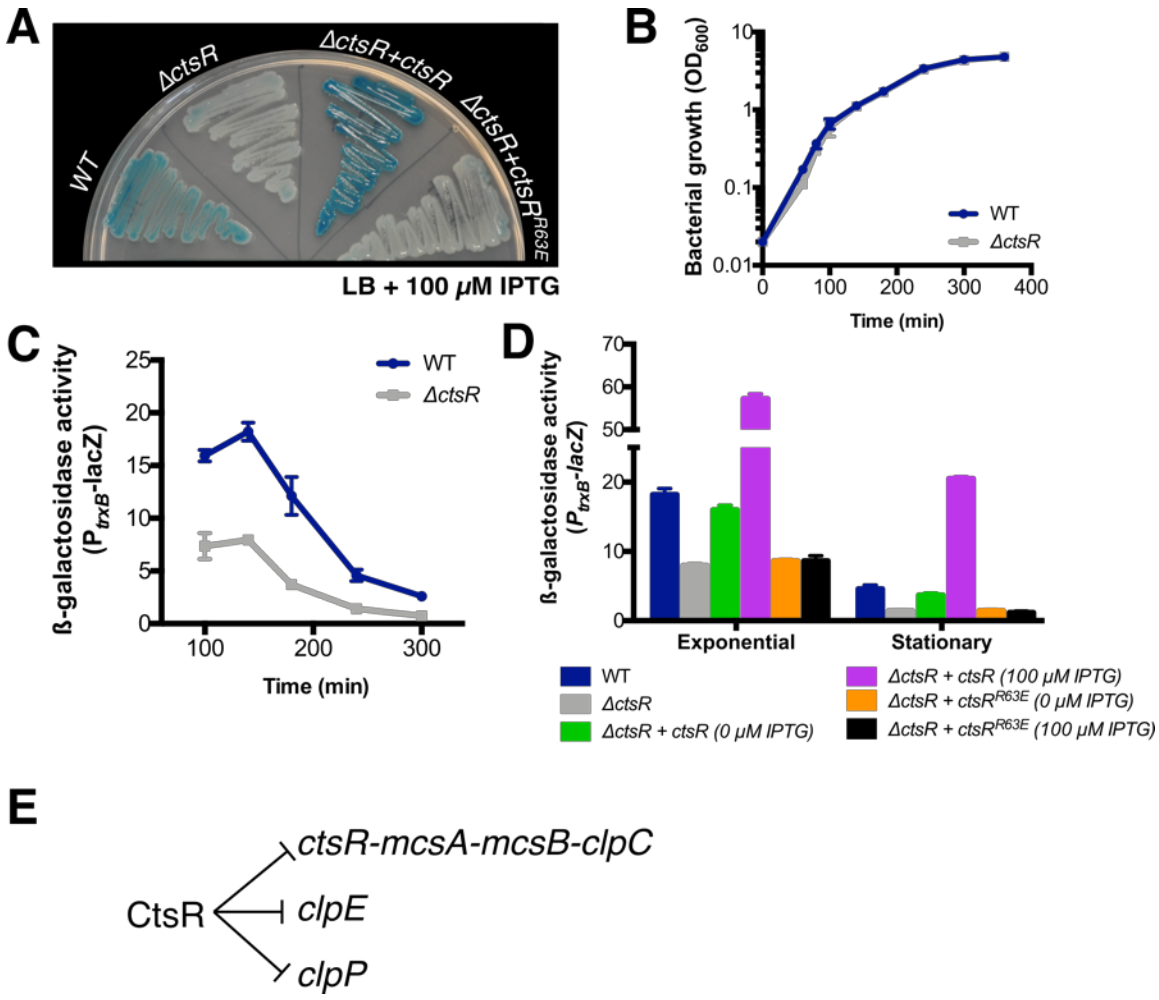

FIG 4.2 DYSREGULATION OF THE CTSR REGULON LEADS TO REDUCED INDUCTION OF P_{TRXB}.

A) Cells lacking CtsR display reduced induction of the Spx-controlled gene *trxB* on LB plates supplemented with X-gal**.** The deletion can be complemented by ectopic expression of *ctsR* but not $ctsR^{R63E}$. B) Growth curve of WT and Δ*ctsR* strains in LB broth. C) Induction of the P_{trxB}-*lacZ* transcriptional fusion throughout the exponential and early stationary phases. Cells were grown in LB broth, and bacterial growth monitored by optical density at 600 nm. D) Complementation of Δ*ctsR* with an ectopic *ctsR* allele driven from the P_{hs} promoter restores the WT phenotype, unlike complementation with the allele $ctsR^{R63E}$. E) Diagram of the CtsR regulon in *B. subtilis*.

4.3.3 Cells lacking CtsR display reduced Spx levels

Deletion of CtsR led to an overall decrease in the expression of the P_{trxB} reporter fusion, which was observed in both exponential and stationary phase (Fig. 4.2); for simplicity, we decided to focus

our study on exponentially growing cells, since our work has been primarily focused on the importance of the Spx regulon in the cell wall stress response (Rojas-Tapias and Helmann, 2018a; 2018b). In *B. subtilis*, CtsR represses genes involved in proteolysis, including those encoding the Clp unfoldases (i.e. ClpC and ClpE), as well as the ClpP protease (Fig 4.2E). We therefore sought to determine if the levels of Spx protein were also affected by inactivation of *ctsR*. An overall decrease in the Spx levels was observed in the Δ*ctsR* strain (Fig 4.3A), which correlated with the reduced activity of the P_{trxB} reporter fusion (Fig 4.2C). Since the CtsR regulon is involved in proteolysis, we hypothesized that this decrease in Spx levels is the result of increased proteolysis.

One possibility is that deletion of CtsR leads to an indirect increase in ClpX levels, which would lead to increased Spx proteolysis. Western blot analysis, however, showed no differences in ClpX levels in both WT and Δ*ctsR* (Fig. 4.3A); the levels of ClpC, by contrast, were significantly elevated in the Δ*ctsR* strain, which is consistent with CtsR acting as a repressor of *clpC* transcription (Fig 4.2E). Alternatively, since deletion of CtsR results in increased expression of *clpP* (Fig 4.2E), increased ClpP levels might result in an elevated number of ClpXP complexes, and therefore increased Spx degradation. To define the basis for the Δ*ctsR* phenotypes, we used a strain with conditional expression of *spx* (P_{hs}-*spx*) in order to maintain a fixed *spx* transcription rate, and monitored Spx levels in both WT and Δ*ctsR* cells lacking or not ClpX or YjbH. If changes in ClpXP-dependent Spx proteolysis are responsible for the decrease in Spx levels in cells lacking CtsR, no differences should be observed between WT and Δ*ctsR* in the Δ*clpX* and Δ*yjbH* genetic backgrounds. Interestingly, this was not the case, as cells lacking CtsR still displayed reduced Spx levels (Fig. 4.3B). These results suggest that the decrease in Spx is likely due to increased proteolysis; however, it seems to occur in a ClpXP-independent fashion.

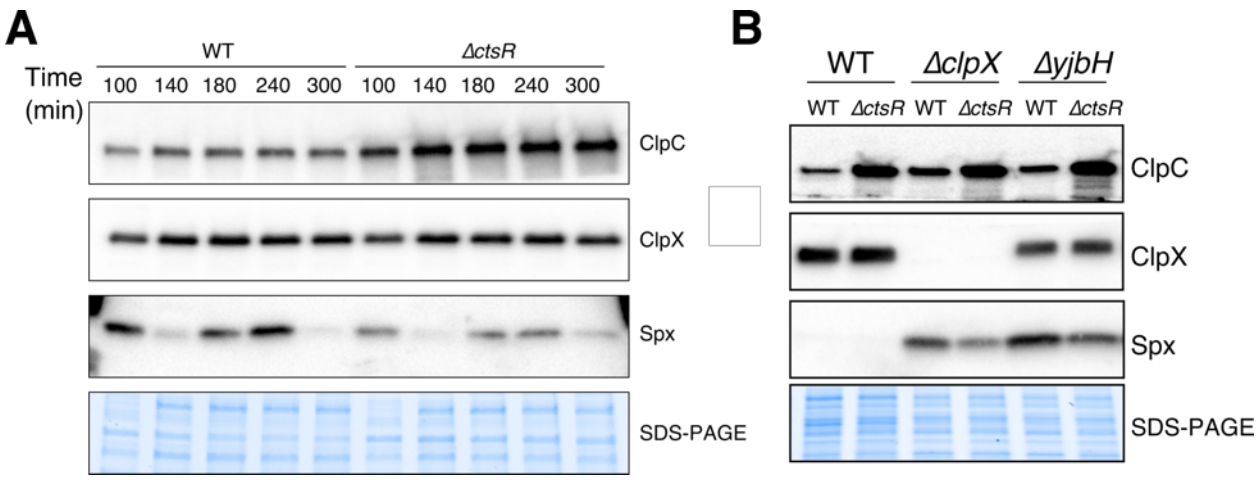

FIG 4.3 CELLS LACKING CTSR DISPLAY REDUCED SPX LEVELS.

A) Spx and ClpX levels in WT and Δ*ctsR* cells throughout exponential and early stationary phase, as assessed using western blot. *B) WT,* Δ*clpX,* and Δ*yjbH* cells featuring conditional expression of *spx* were assessed for Spx levels during the exponential phase. For this, cells were grown up to OD_{600} ~ 0.1 and then IPTG was added to a final concentration of 50 μM to induce *spx* expression. When cells reached OD_{600} = 0.4-0.6, samples were taken, and studied by western blot. ClpC levels were included in both figures to illustrate the derepression of the CtsR regulon in Δ*ctsR* cells. The blots presented are representative of at least three biological replicates.

4.3.4 Spx can be degraded in a ClpX-independent fashion

To explore Spx turnover independent of ClpXP, we monitored Spx turnover in Δ*clpX* cells by using a chloramphenicol chase assay. As expected, Spx was rapidly degraded in the WT strain, which is consistent with the primary role of ClpXP in Spx degradation (Fig. 4.4A). However, Spx was still slowly degraded in Δ*clpX* cells, with an ~40% decrease in Spx over 20 min (Fig. 4.4A). Further evidence for the occurrence of another proteolytic pathway comes from the fact that overexpression of Spx^{DD}, a variant resistant to proteolysis is lethal in WT. We reasoned that if Δ*clpX* cells failed to degrade Spx, overexpression of Spx^{WT} in these cells would also be lethal. However, we observed only a small decrease in plating efficiency (Fig. 4.4B).

A Δ*ctsR* mutant displays increased levels of CtsR-repressed proteins (see ClpC levels in Fig. 4.3), including the ClpCP and ClpEP ATP-dependent proteases (Kruger and Hecker, 1998). To determine if ClpP-dependent proteolysis could account for the reduced Spx levels in the Δ*ctsR* null mutant, we surveyed Spx levels in Δ*clpP* cells with and without CtsR. In this background, the *ctsR* mutation no longer affected Spx levels, indicating that ClpP is responsible for the observed phenotype (Fig 4.4C). We next assessed P_{trxB} activity in Δ*clpP* cells with and without CtsR (as in Fig 4.1). The Δ*clpP* strain was highly unstable on LB plates, and quickly developed suppressor mutations, some of which were mapped to Spx (as monitored by western blot, data not shown). Therefore, we measured P_{trxB}-*lacZ* activity directly on *B. subtilis* colonies obtained through transformation of the *clpX::spec* and *clpP::tet* cassettes into WT and Δ*ctsR* strains. Using this strategy, it is apparent that Δ*ctsR* reduced Spx activity even in cells lacking ClpX, but not in cells lacking ClpP (Fig 4.4D). We conclude that the decrease in Spx in a Δ*ctsR* strain is both independent of ClpX, and dependent on ClpP, thereby implicating the ClpCP and/or ClpEP proteases in Spx degradation.

Spx accumulation in *B. subtilis*, as it is observed in Δ*clpX* or Δ*clpP* mutants, results in reduced growth, sporulation, and competence (Nakano et al., 2001). We therefore reasoned that if deletion of CtsR results in reduced Spx levels, Δ*clpX* cells lacking *ctsR* should display improved growth. To test this hypothesis, we compared the effect of deletion of *ctsR* on bacterial growth, using as a genetic background a *spx* conditional strain. In this background, the growth of WT and Δ*ctsR* was identical regardless of the concentration of inducer (Fig 4.4E), as previously seen (Fig 4.2B). By contrast, in the *spx* conditional cells lacking ClpX, addition of inducer led to a growth defect that was apparent as an extended lag phase (Fig. 4.4E). Interestingly, in this genetic background deletion of *ctsR* improved growth but only when *spx* was induced. A similar result was observed on LB plates (Fig 4.4B). These results suggest that dysregulation of the CtsR regulon alleviates the toxicity imposed by abnormally elevated Spx levels. If ClpEP or ClpCP were involved in this detoxification, deletion of CtsR should have no effect on cells lacking ClpP. This is indeed the case as no differences were observed between WT and Δ*ctsR* in cells lacking the ClpP protease (Fig. 4.4E). Altogether, the present results suggest

that the decrease in Spx levels due to deletion of CtsR is the result of increased ClpEP- and/or ClpCP-mediated Spx proteolysis.

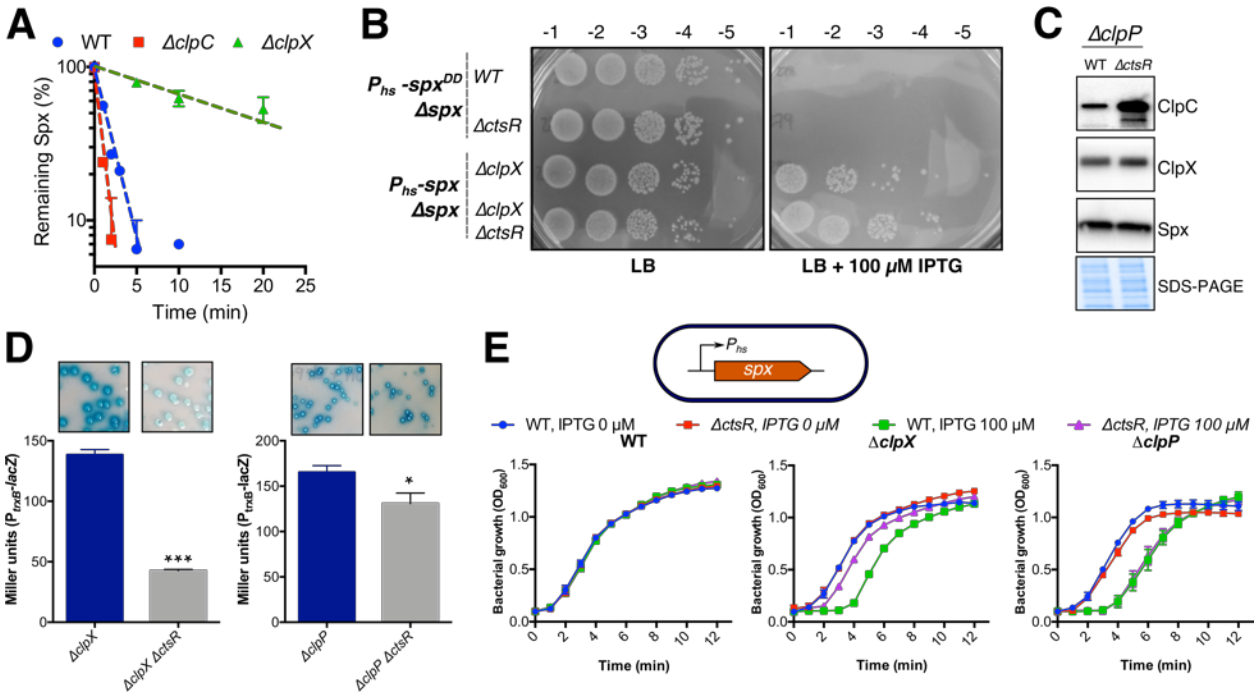

FIG 4.4 SPX IS SUBJECTED TO PROTEOLYSIS IN A CLPXP-INDEPENDENT FASHION

A) A protein chase assay was carried out to survey Spx stability in the WT, Δ*clpC*, and Δ*clpX* null mutants under unstressed conditions. B) Overexpression of *spx* in Δ*clpX* cells do not phenocopy overexpression of spx^{DD} as observed in spot dilution assays. C) Δ*clpP* and Δ*clpP* Δ*ctsR* cells featuring conditional expression of *spx* were assessed for Spx levels during the exponential phase as described in Fig 4.3B. D) The activity of the P_{trxB} promoter was studied in cells lacking the ClpX and ClpP proteins. Beta-galactosidase activity was studied directly on bacterial colonies grown for two days on LB plates. A section of the plate is shown to illustrate the differences in P_{trxB} expression of the transformant strains growing on LB plates supplemented with X-gal, as shown in Fig 4.2A. This experiment was performed in triplicate. A t-test was performed to compare the mean value of each pair of strains. *, **, *** indicate significant differences with $p<0.05$, $p<0.01$, and $p<0.001$, respectively. E) Growth curves of the *spx* conditional strain in WT, Δ*clpX*, and Δ*clpP* backgrounds in absence or presence of IPTG to induce *spx* expression. The cartoon illustrates the *spx* conditional strain.

4.3.5 ClpXP-independent Spx degradation is mediated by ClpCP

To define the protease(s) responsible for Spx degradation, we monitored the stability of Spx in Δ*clpX,* Δ*clpC* Δ*clpX*, Δ*clpE* Δ*clpX, and* Δ*clpP* mutants featuring conditional expression of Spx. By using cells lacking distinct pairs of Clp unfoldases, we sought to determine the contribution of ClpC and ClpE to Spx degradation in the Δ*clpX* mutant cells. While the degradation rate was similar in both Δ*clpX* and Δ*clpX* Δ*clpE* cells, cells lacking both *clpX* and *clpC* were unable to degrade Spx over the course of the experiment (Fig. 4.5). In cells lacking *clpP*, as expected, no degradation was observed. Therefore, ClpCP contributes to Spx degradation *in vivo*. Indeed, previous biochemical experiments demonstrated that ClpCP is competent to degrade Spx *in vitro* (Nakano et al., 2002b). We also observed that the double Δ*clpX* Δ*clpC* mutant strain exhibited reduced Spx levels compared with Δ*clpX* or Δ*clpP* (Fig 4.5). Sanger sequencing of the obtained clones, however, showed no mutations within P_{hs} or *spx*. Similarly, Illumina sequencing of the entire genome showed no mutations in the genes known to be involved in Spx regulation. At this point, it is therefore unclear what led to reduced Spx levels.

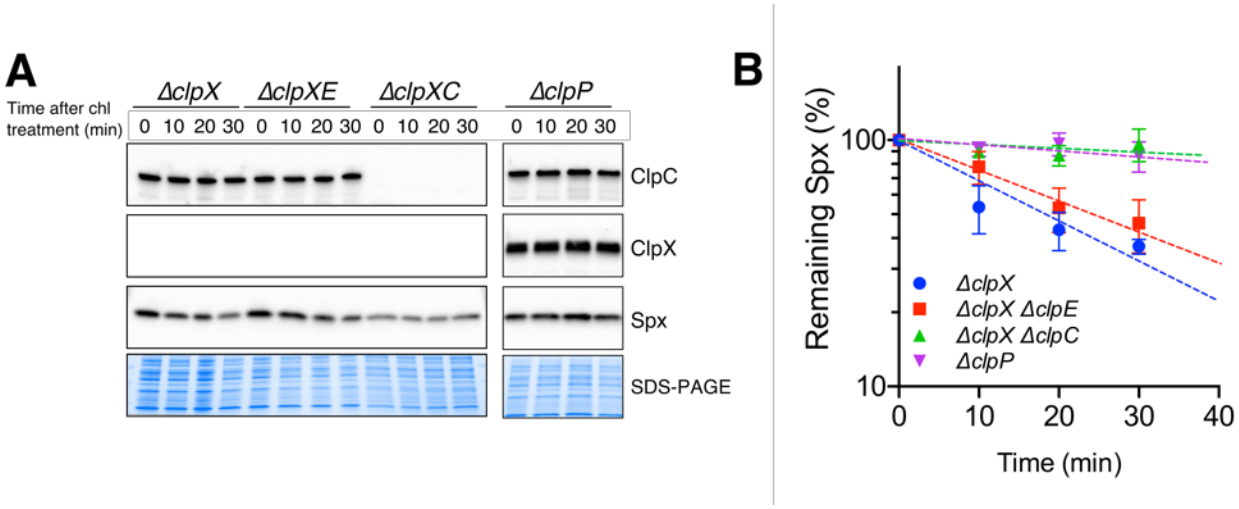

FIG 4.5 CLPCP IS CAPABLE OF DEGRADING SPX IN VIVO.

The stability of Spx was studied in cells with conditional expression of *spx* lacking ClpX, ClpX and ClpE, ClpX and ClpC, and ClpP using a protein chase assay. A representative blot is shown in A), while quantitated and normalized data of at least three independent experiments are presented in B). Spx levels were normalized using the quantitation of total proteins in the SDS-

PAGE gel and of bands in the western blot by densitometry. Time zero, before chloramphenicol addition, was considered to be 100%. Error bars indicate standard error of the mean (SEM).

4.3.6 MecA is important to prevent Spx toxicity in Δ*clpX* cells

In *B. subtilis*, three adaptor proteins for ClpC are known: MecA, McsB, and YpbH. To examine the role of these adaptors in Spx proteolysis, we used a Δ*clpX* background (to eliminate the ClpXP protease activity) and constructed strains lacking each of these adaptors to see if they phenocopied a Δ*clpX* Δ*clpC* double mutant. [For construction of Δ*clpX* Δ*mcsB* and Δ*clpX* Δ*ypbH*, gDNA of Δ*clpX* cells was transformed into Δ*mcsB* and Δ*ypbH* strains. While for construction of the Δ*clpX* Δ*mecA* strain, we used gDNA from a Δ*mecA* strain, and as recipient a Δ*clpX* strain with conditional expression of *spx.* We chose this latter strategy since both Δ*clpX* and Δ*mecA* strains are not genetically competent.] During these studies, we observed that *clpX* and *mecA* were synthetic lethal, suggesting that MecA may be the primary adaptor required for ClpCP-mediated Spx proteolysis, which is apparently essential in cells unable to degrade Spx through the ClpXP pathway. Synthetic lethality is evident from the IPTG-dependent growth of a P_{spac}-*clpX* Δ*mecA* strain. Of note, this synthetic lethality disappears in an *spx* mutant strain (Fig 4.6B).

These results suggest that the synthetic lethal relationship between *mecA* and *clpX* is due to the accumulation of toxic levels of Spx if both of these genes are absent. Support for this notion is provided from genetic studies. We hypothesized that the difficulty in construction of a Δ*clpX* Δ*mecA* strain might be alleviated in a recipient lacking *spx*. We therefore used a *clpX::spec spx::tet* strain, which is genetically competent, as recipient and either *mecA::ery* genomic DNA (gDNA) as donor for transformation. In this experiment, relatively few transformants were recovered, which we suspected might be due to the close proximity of *mecA* and *spx* (Fig 4.6C), which could frequently result in re-introduction of *spx* into strains now lacking *clpX* and *mecA*. Indeed, the only cells recovered in this transformation were those that retained the *spx*::*tet* allele of the recipient. In contrast, if the donor DNA also lacked the spx gene (*mecA::ery spx::kan*) it was now possibly to construct the desired would

mecA clpX double mutant with ~20x higher transformation efficiency (Fig. 4.6A). These results are reminiscent of the toxicity observed upon Spx^{DD} overproduction, and imply that MecA is important to prevent Spx overaccumulation. This role is supported by *in vitro* data that showed that MecA and YpbH can assist Spx degradation through ClpCP (Nakano et al., 2002b).

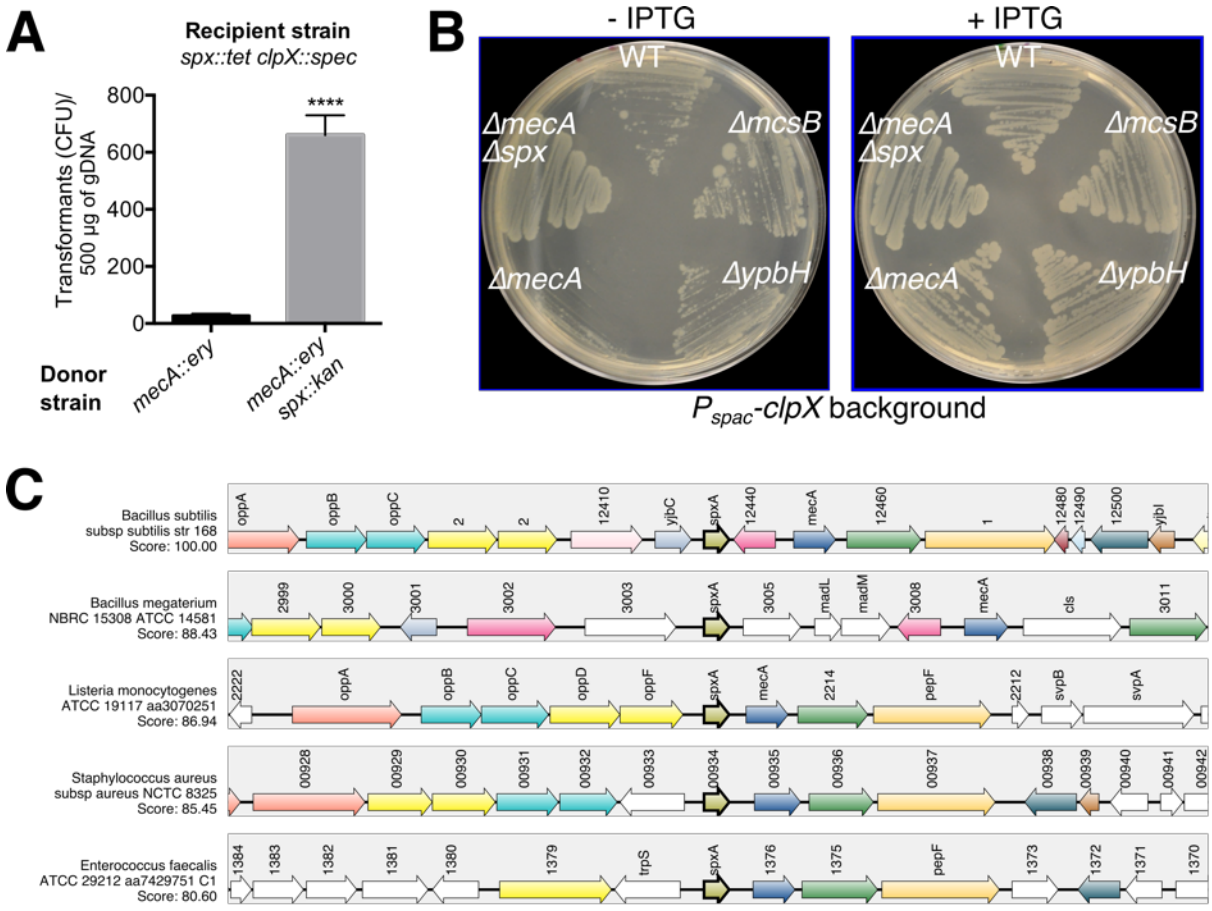

FIG 4.6 CELLS LACKING CLPX AND MECA ARE NOT VIABLE. OVEREXPRESSION OF CLPC IN ΔCLPX CELLS DOES NOT PHENOCOPY THE DELETION OF CTSR

A) Number of colonies after transformation of the *spx::tet clpX::spec* recipient strain with 500 ng of gDNA isolated from the donor strains 1) *mecA::ery* and 2) *mecA::ery spx::kan.* This experiment was performed in triplicate. A t-test was performed to compare the mean value on both strains. *, **, *** indicate significant differences with $p<0.05$, $p<0.01$, and $p<0.001$, respectively. B) Growth of a *clpX* depletion strain lacking or not *mecA*, *mcsB*, *ypbH*, and both *mecA* and *spx* on LB plates supplemented or not with 100 µM IPTG. Cells were initially grown on LB plates supplemented with 100 µM IPTG, then subcultured on LB plates without IPTG in order to deplete ClpX, and finally streaked on LB plates with either 0 µM or 100 µM

IPTG (as shown in the picture). C) Analysis of synteny of the *spx* gene throughout several Firmicutes reveals genetic proximity to *mecA.* The *mecA* gene is displayed in dark blue. This bioinformatic analysis was performed using the SyntTax software (Oberto, 2013).

4.3.7 McsA and McsB are required for Spx degradation upon ClpC overexpression

MecA is critical for Spx degradation and/or stabilization in unstressed cells lacking ClpX. However, whether MecA is responsible for the lower Spx levels observed in Δ*ctsR* cells, where *clpC* is highly expressed, is unknown. To explore this idea, we studied whether overexpression of *clpC* alone may improve the plating efficiency of a Δ*clpX* strain.

Previously, we had noted that the plating efficiency of *B. subtilis* cells was dramatically affected by Spx levels. For instance, when cells with conditional expression of *spx* were transformed with the *clpX::spec* or *clpP::tet* cassettes, the plating efficiency decreased ~20- and 50-fold by addition of IPTG; importantly, this phenotype disappeared in cells lacking Spx. We then used this approach to study whether overexpression of *clpC* alone was sufficient to mediate Spx degradation. When either the *clpX::spec* or *clpX::kan* cassettes were moved into a strain with conditional expression of *clpC* (i.e. Δ*clpC amyE::*P_{hs}-*clpC*), no differences in plating efficiency were observed between plates supplemented or not with IPTG (Fig 4.7A). This result thus indicates that *clpC* alone cannot alleviate Spx toxicity. Next, we placed the *mcsA-mcsB-clpC* operon under IPTG control using a pMUTIN based system (i.e. P_{spac}-*mcsA-mcsB-clpC,* see experimental procedures). When the *clpX::kan* cassette was transformed into this strain, we noticed that the plating efficiency positively correlated with the concentration of IPTG, thereby suggesting that upregulation of the whole *clpC* operon is required to alleviate Spx toxicity. Here, the plating efficiency was only determined by Spx, as these differences were no longer observed in cells lacking the *spx* gene (Fig 4.7B). Over the course of this study, we noticed differences in terms of survival when different antibiotic resistance cassettes (i.e. translation inhibitors) were used to inactivate either *clpX* or *clpP* in cells with elevated Spx levels (see Fig 4.7A). The molecular bases for this phenomenon, however, remain unknown. Altogether, the present results

indicate that the arginine kinase system McsA/McsB is also required for Spx degradation, but only under conditions that result in *clpC* upregulation.

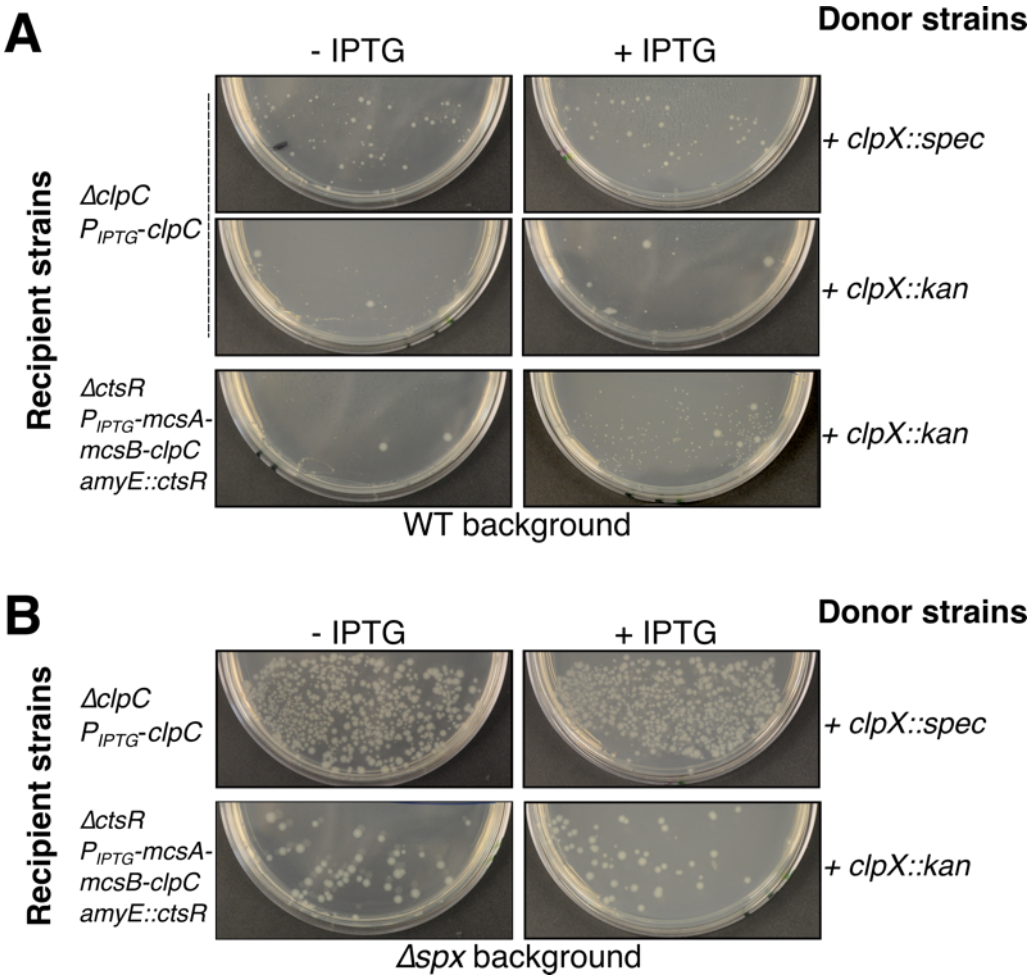

FIG 4.7 OVEREXPRESSION OF MCSA-MCSB-CLPC POSITIVELY AFFECTS SURVIVAL OF ΔCLPX CELLS IN A SPX-DEPENDENT FASHION

A) Transformation experiments were performed to determine the effect of *clpC* or *mcsA-mcsB-clpC* overexpression on the platting efficiency of *clpX* cells. For this, cells were transformed with 250 ng of donor DNA, and then the transformants were spotted on plates containing 0 μM or 100 μM IPTG. The promoters used to drive *clpC* and *mcsA-mcsB-clpC* expression were not fully identical, as those transcriptional units were constructed using the pPL82 and pMUTIN systems, respectively. Nevertheless, in both cases they are responsive to IPTG addition. B) An identical experiment as the one described in Fig 4.6B was performed on otherwise isogenic strains lacking *spx*. Each experiment was performed at least three times with similar results, and the shown plates are representative of those replicates.

4.3.8 Role of arginine phosphorylation on Spx degradation

Our transposon mutagenesis screening also uncovered the YwlE arginine phosphatase as another possible modulator of Spx activity/stability in growing cells. As expected, cells with a clean deletion of *ywlE* also displayed reduced induction of P_{trxB} in growing cells, and this effect was abolished in a Δ*mcsB* Δ*ywlE* double knockout (Fig 4.8A). The present results are therefore suggestive of a role for arginine phosphorylation in Spx regulation. To explore whether arginine phosphorylation affects Spx turnover through ClpCP, we studied the effect of *ywlE* deletion on Spx stability in Δ*clpX* cells. Results showed increased Spx proteolysis in cells lacking YwlE, but this phenotype was not abolished in the Δ*mcsB* Δ*ywlE* double mutant. (Fig 4.8B). This result might be explained by a possible compensatory effect mediated by MecA on YpbH on ClpC activity. To further determine if arginine phosphorylation was important for Spx degradation, we monitored the growth of WT and Δ*ywlE* in cells featuring conditional *spx* expression and lacking ClpX (as seen in Fig 4.4E). In these strains, when *spx* was uninduced, the Δ*ywlE* strain only displayed a modest defect in growth compared with its WT counterpart. Interestingly, upon addition of inducer, the deletion of *ywlE* dramatically affected growth, as cells displayed a shorter lag phase compared with WT (Fig 4.8C). Deletion of *ywlE* thus phenocopied the effect of deletion of *ctsR* as observed in Fig 4.3E (Fig 4.8C). Altogether, these results suggest that arginine phosphorylation is important for Spx degradation, however, whether this effect is direct or indirect remains unclear.

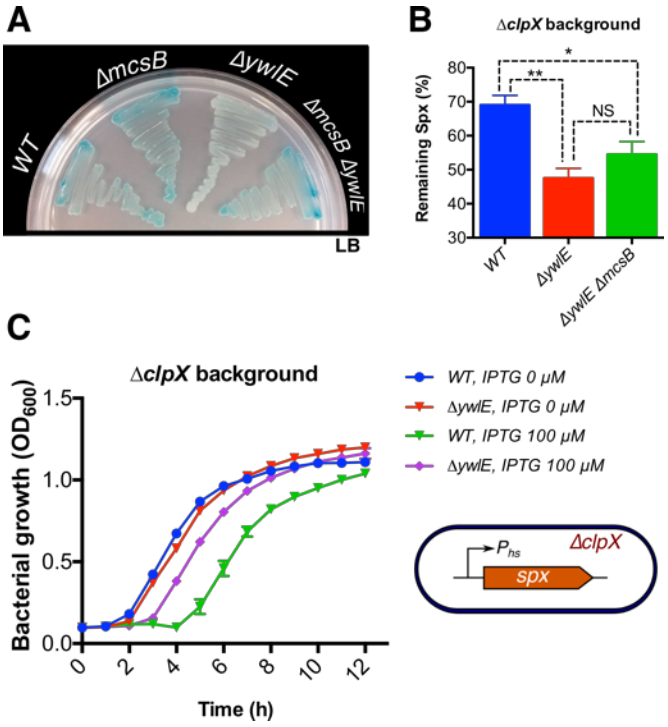

FIG 4.8 ARGININE PHOSPHORYLATION AFFECTS SPX REGULATION

A) Cells lacking *ywlE* display reduced induction of the *trxB* promoter, and this phenotype is alleviated by deletion of *mcsB*. B) Protein chase experiment show that *ywlE* deletion results in increased Spx proteolysis, however, this phenotype was not fully rescued by deletion of *mcsB*. An ANOVA test was performed to compare Spx degradation in the different strains after 30 min of chloramphenicol chase. Multiple comparisons was carried out using the Tukey's HSD test. * and ** indicate significant differences with $p<0.05$ and $p<0.01$, respectively. C) Growth curves of Δ*clpX* strains featuring IPTG-dependent expression of *spx* (as shown in the cartoon), and lacking or not the YwlE arginine phosphatase. Cells were subcultured beforehand in absence of IPTG, and then grown in presence of 0 μM or 100 μM IPTG to drive *spx* expression.

4.3.9 ClpCP-mediated Spx degradation is important under disulfide stress

The present results suggest that ClpCP might be important for Spx degradation under conditions that result in upregulation of the CtsR regulon. Such conditions are observed, for instance, under disulfide, heat, and ethanol stress (Nicolas *et al.*, 2012). Interestingly, those conditions also reduce Spx proteolysis due to inactivation of ClpX and/or YjbH (Engman and Wachenfeldt, 2015; Garg et al., 2009). It is therefore likely that ClpCP-mediated Spx degradation has evolved as a mechanism

to prevent the negative effects of Spx accumulation under conditions wherein Spx degradation through ClpXP is dramatically reduced. To determine if this is the case, we monitored Spx degradation in WT, Δ*clpX*, and Δ*clpC* cells upon exposure to diamide. While Spx was slowly degraded in WT and Δ*clpX* cells, no degradation was observed in cells lacking Δ*clpC* (Fig 4.9). Thus, under disulfide stress ClpCP is important to degrade Spx.

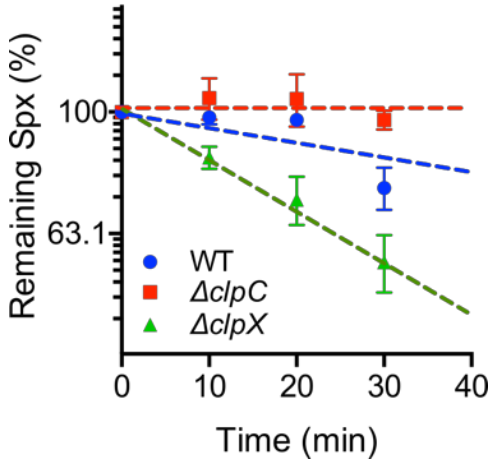

FIG 4.9 SPX DEGRADATION UNDER DISULFIDE STRESS IS MEDIATED BY CLPCP.

A protein chase assay shows that cells lacking ClpC are unable to degrade Spx in response to diamide. Each experiment was performed in triplicate. Bars mean SEM.

4.3.10 Spx regulates the expression of the *ctsR* operon

Using ChIP-tilling microarray technology, Rochat et al. (2012) showed that Spx binds to and affects the expression of the *ctsR* promoter. This interaction, however, was not explored in further detail. To assess the contribution of Spx for induction of the autoregulated *ctsR* operon, we studied by northern blot the *clpC* mRNA levels in WT, Δ*ctsR*, Δ*spx*, and Δ*ctsR* Δ*spx* cells in response to vancomycin, a potent inducer of the Spx regulon (Rojas-Tapias and Helmann, 2018b). Vancomycin led to induction of the *ctsR* operon, and this activation was suppressed in Δ*spx* cells, which confirms the role of Spx in activation of the *ctsR* regulon. Deletion of *ctsR* resulted in derepression of the *ctsR* operon, which was observed as a more intense band at the initial time point and is consistent with

negative autoregulation. This deletion, however, did not abolish the activation of the operon in response to stress (Fig 4.10). Conversely, deletion of Spx largely suppressed this activation, suggesting that both CtsR and Spx regulate the expression of the *ctsR* operon. As reference, we studied another CtsR-regulated gene (i.e. *clpE*), which was also reported to be under Spx control, however, no contribution of Spx to *clpE* expression was observed (Fig 4.10). Taken together, the present evidence demonstrates the existence of a feedback loop between two major regulators CtsR and Spx, in which the interaction between both transcriptional factors is likely important to fine-tune various stress responses.

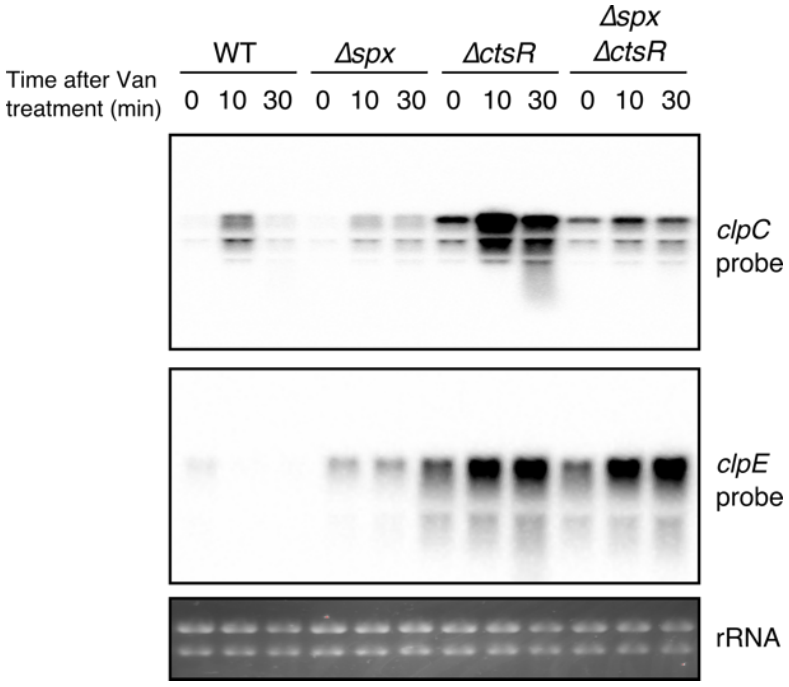

FIG 4.10 BOTH CTSR AND SPX DRIVE THE EXPRESSION OF THE CTSR OPERON

Northern blot shows the expression profile of two CtsR-regulated genes in response to vancomycin. *clpC* and *clpE* mRNA levels were studied in different genetic backgrounds. This experiment was performed in duplicate with identical results.

4.4 Discussion

Regulation of Spx in *B. subtilis* is complex and involves many regulatory mechanisms. Activation of the Spx regulon in response to both disulfide and cell wall stress, for instance, has been recently shown to occur through largely independent pathways (Rojas-Tapias and Helmann, 2018b). Induction of the Spx regulon under disulfide stress involves the aggregation of the adaptor protein YjbH, a decrease in the activity of the ClpXP protease, and oxidation of the Spx protein, which eventually leads to Spx accumulation and activation of the regulon (Engman and Wachenfeldt, 2015; Nakano et al., 2005; Zhang and Zuber, 2007). By contrast, under cell wall stress, transcriptional induction of the *spx* gene through a σ^M-dependent promoter and stabilization by the anti-adaptor protein YirB are both required for full and timely activation of Spx-controlled genes (Rojas-Tapias and Helmann, 2018a; 2018b). Although the regulatory mechanisms that result in induction of the Spx regulon under disulfide and cell wall stress are fairly well understood, some of the phenotypes observed throughout the course of our experiments suggested that additional regulatory mechanisms were still at play.

In order to find additional regulators of Spx activity or stability, we carried out transposon mutagenesis to identify genes that affected induction of the Spx-controlled gene *trxB*. This analysis identified *ctsR* as a potential regulator of Spx activity. CtsR is a protein repressor that prevents the expression of the Clp genes (i.e. *clpE*, *clpC*, and *clpP*), the arginine phosphorylation system (i.e. *mcsA* and *mcsB*), and its own transcription; yet, how deletion of CtsR impacted the activity of Spx was unclear (Fig 4.1). We thus reasoned that analysis of this mutant would provide a further understanding of the regulatory mechanism that govern Spx activity.

Cells lacking CtsR displayed reduced P_{trxB} activity and Spx levels (Fig 4.2). Initially, we hypothesized that deletion of CtsR might result in increased ClpX levels, which in turn would lead to increased Spx proteolysis. This was not the case though, as ClpX levels were similar in WT and *ΔctsR*.

Interestingly, in cells lacking ClpX, deletion of *ctsR* also resulted in reduced P_{trxB} activity and Spx levels. The same phenotype was observed upon deletion of *yjbH*, which encodes the adaptor protein required for ClpXP-dependent degradation of Spx. These results therefore suggested that Spx proteolysis might occur through an alternative pathway (Fig 4.3). Since deletion of CtsR resulted in reduced Spx levels even in cells lacking ClpX, we reasoned that this phenotype should also be ClpP independent. Surprisingly, deletion of ClpP led to similar levels of induction of both reporter fusions in WT and *ΔctsR,* as well as similar Spx levels (Fig 4.4). This observation led as to think that the observed phenotype in *ΔctsR* cells was due to ClpX-independent Spx proteolysis.

By using genetic experiments, we showed that Spx is also subjected to degradation via ClpCP (Fig 4.5), which was consistent with previous *in vitro* experiments (Nakano et al., 2002b). Interestingly, we noted that the construction of cells lacking both *clpX* and *clpC* was extremely challenging, and also that deletion of *spx* suppressed this phenotype. This result suggested that Spx was highly toxic in cells lacking both unfoldases, as it occurs in cells lacking *clpP* (Fig 4.4D). Analysis of the adaptor proteins involved in Spx degradation via ClpCP uncovered the synthetic lethality of *mecA* and *clpX*, and also that this lethality depends on the presence of Spx (Fig 4.6). MecA is the major adaptor protein for ClpCP in absence of stress, and some of the pleiotropic phenotypes due to ClpC deletion are mimicked by inactivation of MecA (e.g. competence, sporulation) (Kirstein et al., 2009; Mogk and Bukau, 2003; Turgay et al., 1998). One possibility is that MecA assists Spx degradation through the ClpCP protease, thus reducing Spx levels, which is consistent with *in vivo* and *in vitro* data. Alternatively, the MecA-Spx-ClpC complex, previously reported (Nakano et al., 2002a), might inactivate a fraction of Spx, and thus prevent its toxicity. This latter hypothesis is indeed supported by the fact that unlike the *ΔmecA ΔclpX* double knockout mutant, deletion of *clpP* is detrimental to cells but not lethal (compare results in Fig 4.4D and Fig 4.6A). A similar model for inactivation of a transcription factor has been shown for Spo0A, wherein both MecA and ClpC modulate Spo0A activity

by direct physical interaction (Tanner et al., 2018). The linkage between Spx and MecA seems to be supported by the genetic proximity of both genes along many Firmicutes (Fig 4.6C). However, the precise role of MecA in Spx regulation remains to be determined.

Overexpression of *clpC* had no effect on the plating efficiency of *ΔclpX* cells, suggesting that increased ClpC levels alone do not likely account for the phenotype of *ΔctsR* cells (Fig 4.7). Overexpression of the *mcsA-mcsB-clpC* operon, by contrast, had a positive effect. This beneficial effect due to *mcsA-mcsB-clpC* overexpression was mapped to Spx, since no differences in plating efficiency were observed in otherwise isogenic strains lacking *spx*. We chose this experimental approach (i.e. platting efficiency after genetic transformation) since cells with conditional expression of *clpC,* the *clpC* operon, or *mecA* in a *clpX* background were highly unstable (as observed in *ΔclpP* mutants). Results thus showed that upregulation of *mcsA* and *mcsB* is also required to alleviate the toxic effects caused Spx accumulation, and suggests a role for arginine phosphorylation in Spx regulation (Fuhrmann et al., 2009; Kirstein et al., 2007). These observations are also supported by our transposons screening that uncovered the YwlE arginine phosphatase as a possible modulator of Spx activity/stability, and seem to imply that elevated arginine phosphorylation is required for Spx detoxification. Protein chase experiments further showed that Spx proteolysis is increased in cells lacking YwlE, which is consistent with this hypothesis (Fig 4.8). Remarkably, arginine phosphorylation of Spx and MgsR, an Spx paralog, was previously detected in a study that also uncovered the role of arginine phosphorylation in ClpCP-mediated protein degradation (Trentini *et al.*, 2016). A total of four residues were found to be modified in Spx, including Spx^{R14}, Spx^{R91}, Spx^{R100}, and Spx^{R112}; and two in MgsR, including $MgsR^{R17}$ and $MgsR^{R95}$. Interestingly, the Spx^{R14} and $MgsR^{R17}$ residues are identically located in the tertiary structure of both Spx paralogues, and the residues Spx^{R91} and $MgsR^{R95}$ are contiguous (Fig S1). Altogether, the present evidence is suggestive of direct phosphorylation of Spx and degradation through ClpCP under conditions that result in upregulation of the CtsR regulon.

Spx turnover in absence of ClpX occurred slowly, and the Spx half-life was estimated to be >20 min (compared to <2 min by ClpXP) (Fig 4.4A, Fig 4.5). The importance of Spx degradation through ClpCP thus is unclear. We hypothesized that ClpCP might be important under conditions that result in inactivation of ClpXP, such as disulfide stress. In support of this hypothesis, we observed ClpCP-mediated Spx degradation in response to treatment with diamide (Fig 4.9). This slow degradation rate also presents an alternative scenario in which the formation of the MecA-Spx-ClpC complex, as previously reported (Nakano et al., 2002a), might be important for Spx stabilization in unstressed conditions. Consistently, cells lacking ClpC displayed reduced Spx half-life compared with WT (Fig 4.4A). Moreover, we observed that only a fraction of Spx could be degraded via ClpCP (Fig 4.4A, Fig 4.5). This suggests that either not all Spx can be degraded through ClpCP, or that our experimental setup affected Spx turnover via ClpCP. The adaptor MecA, for instance, is simultaneously degraded with the substrate, and therefore chloramphenicol addition is expected to affect MecA synthesis and therefore substrate degradation.

Although a role for Spx in activation of the *ctsR* regulon was previously suggested (Trentini et al., 2016), this interaction was not explored in further detail. Here, we show that both CtsR and Spx drive the expression of the *ctsR* regulon through negative and positive action on the *ctsR* promoter, respectively (Fig 4.10). This interaction implies that the fine-tuning of these regulators is important to deliver an appropriate response against stressful conditions. Furthermore, this result implicates that Spx also drives the expression of the genes encoding the arginine phosphorylation system, and therefore that stressful conditions that activate Spx might result in the selective degradation of proteins through ClpCP. The interplay between Spx and CtsR also leads to formation of a negative feedback loop for Spx. Further studies are, however, required to elucidate the contribution of both regulators in the activation of the CtsR regulon.

4.5 Experimental procedures

4.5.1 Bacterial strains and culture conditions

All bacterial strains are listed in Table S1. *Bacillus subtilis* strains (all based on the *B. subtilis* 168 wild-type) were grown under standard conditions: lysogeny broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) broth at 37 ºC with vigorous shaking, unless otherwise stated. *Escherichia coli* DH5α was used for plasmid construction. Antibiotics were added to the growth medium when appropriate: 100 µg ml^{-1} ampicillin for *E. coli*, and 1 µg ml^{-1} erythromycin plus 25 µg ml^{-1} of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 µg ml^{-1} chloramphenicol, 100 µg ml^{-1} spectinomycin, and 10 µg ml^{-1} kanamycin for *B. subtilis*.

4.5.2 Cloning and site-directed mutagenesis

For cloning purposes, the PCR reactions were performed using the high-fidelity Phusion DNA Polymerase (NEB) following manufacturer's instructions, and genomic DNA of *B. subtilis* [HB18501] unless otherwise stated. The primers used in this study are listed in Table S2. For overlap PCR, one first round of PCR without primers was performed using the following program: initial denaturation (98ºC x 1'), 5 cycles of assembly (98ºC x 10'', Tm+5ºC x 40'', and 72ºC x 30'' per kb''), 5 cycles of assembly (98ºC x 10'', Tm ºC x 40'', and 72ºC x 30'' per kb'), and 5 cycles of assembly (98ºC x 10'', Tm-5ºC x 40'', and 72ºC x 30'' per kb). Equimolar amounts of both DNA fragments were used (~50 ng of the smallest fragment) for assembly. Then 3 µl of the resulting product was used for conventional PCR amplification; the PCR reactions were performed as recommended by the manufacturer. For deletion of the erythromycin or kanamycin cassettes in the strains from the Bacillus Genomic Stock Center (BGSC) the pDR244 plasmid was used.

For construction of the *spx* allele under control of the hy-spank promoter [i.e. P_{hs}-*spx (cm)*], the PCR product obtained with primers DR445 and DR446 was used. For construction of the IPTG-inducible *ctsR* allele [i.e. P_{hs}-*ctsR (cm)*], the fragment containing the *ctsR* gene was amplified using the primers DR315 and DR316. To generate the $ctsR^{R63E}$ allele under IPTG control [i.e. P_{hs}-$ctsR^{R63E}$ *(cm)*], the mutagenic primers DR345 and DR346 were additionally used, and the resulting PCR products were ligated by overlap PCR. The resulting fragments of *spx*, *ctsR*, and $ctsR^{R63E}$, as well as the pPL82 vector, were digested with XmaI and XbaI, ligated using the T4 ligase, and transformed into *E. coli* DH5a. The generated vectors were used to transform *B. subtilis*.

For construction of the *amyE::ctsR* (cm) complementation cassette, a fragment covering the *ctsR* coding region and its promoter were amplified using primers DR450 and DR451, purified, and digested with EcoRI and BamHI. The resulting PCR product was cloned into pDG1662, which was digested using the same restriction enzymes. Both insert and vector were ligated using T4 ligase, and transformed into *E. coli*. The generated vector was used to transform *B. subtilis*.

For construction of P_{spac}-*clpX*, we amplified the N-terminal region of *clpX* with two sets of primers: first, we used primers DR166 and DR366, and then DR365 and DR342. The goal was to eliminate an internal HindIII restriction site. The resulting PCR products were ligated by overlap PCR. For construction of P_{spac}-*mcsA*-*mcsB*-*clpC*, we amplified by PCR the region encompassing the *ctsR* ribosome-binding site and the N-terminal region of *mcsA* using as template genomic DNA from the Δ*ctsR* strain [HB18984]. The primers used were DR343 and DR364. The resulting strain hence featured conditional expression of the *mcsA*-*mcsB*-*clpC* operon, and a markerless deletion of *ctsR*. The resulting fragments, as well as the pMUTIN-Spec vector, were digested with HindIII and KpnI. Ligated using T4 ligase, and transformed into *E. coli* DH5α. The resulting plasmids were transformed into *E. coli* TG1 to generate multimeric plasmids, and then 1 µg of plasmid DNA used to transform *B. subtilis*.

4.5.3 Transposons library

B. subtilis cells with the appropriate genotype were transformed with the pMarA plasmid, plated onto LB + 0.3 mg ml^{-1} erythromycin, and incubated at 28ºC for 48 h. The resulting transformants were stored at -80ºC (host for transposition). Cells containing the pMarA plasmid were then grown overnight at 28ºC in LB broth supplemented with kanamycin and erythromycin. A new culture (1:40) was started in LB broth + kanamycin (to select for the transposition events) and incubated for 4 h at 28ºC, the cells were then transferred at 37ºC and incubated for three more hours, and finally plated on LB amended with 15 µg ml^{-1} kanamycin and 0.2 mg ml^{-1} X-gal. Plates were incubated at 42ºC for loss of the plasmid, and then candidate mutant colonies were selected on the basis of the intensity of its blue color. Whiter colonies on the plates were chosen, subjected to one more round of selection, and finally 30 clones from each library saved for further studies. To determine the site of *mariner* insertion, chromosomal DNA was isolated using the DNeasy Kit (Qiagen), digested with the Taqα1 restriction enzyme, and the products ligated using the T4 ligase. The resulting DNA was used as template for an inverse PCR reaction using the primers 6299 and 6300 annealing the *mariner* transposon. The PCR products were in-column cleaned, and analyzed by sequencing using the 6301 internal primer. The sequencing information was then used to map the transposon insertion site.

4.5.4 β-galactosidase activity

The cells were grown until OD_{600} reached ~0.5. Then, cells were treated or not with different chemicals, and incubated at 37ºC with agitation. After specific time points, samples were taken, washed twice in PBS, and finally resuspended in 900 µl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, $MgSO_4 \cdot 7H_2O$) supplemented with 400 µM DTT. Alternatively, several colonies were recovered from the plate and resuspended in PBS at OD_{600}~0.5, washed in PBS, and resuspended in 900 µl of Z buffer + 400 µM DTT for further experiments. Optical density at 600 nm

was measured, and then the cells were lysed using 100 µg ml^{-1} lysozyme at 37ºC for 30 min. Next, 200 µl of 4 mg ml^{-1} ONPG were added to the lysate, and the reaction was incubated at 28ºC until the samples produced a visible yellow color. The reaction was stopped by adding 500 µl of 1.0 M Na_2CO_3. The absorbance was then measured at 420 nm and 550 nm, and β-galactosidase activity was determined using the following equation: Miller Units = $1000*[OD_{420}-1.75*OD_{550}]/(t*v*OD_{600})$, where *t* is time in minutes and *v* is the volume of culture used in the reaction.

4.5.5 Western Blot

A total of 5 ml of cells were collected, washed in PBS, and resuspended in 150 µl of disruption buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% glycerol) supplemented with the cOmplete™ EDTA-free Protease Inhibitor Cocktail. The cells were disrupted by sonication, and then centrifuged for 15 min at 13,500 rpm at 4ºC. The soluble fraction was collected and quantified using the Bradford Assay. Reducing sample buffer was added to the protein extract, and then 5 µg of protein were loaded in a 4-20% SDS-PAGE. Proteins were transfer onto a PVDF membrane using the TransBlot Turbo Transfer System (Bio-Rad, USA). The membrane was blocked using 5% protein blotting blocker dissolved in TTBS for 1 h. at RT. Then, the primary antibodies were resuspended in 0.5% protein blotting blocker dissolved in TTBS and incubated for 16 h at 4ºC. Finally, an anti-rabbit HRP-conjugated secondary antibody was added and incubated for 2 h at RT. The membrane was revealed using the Clarity Western ECL substrate and visualized in a Gel documenter. For quantification of Spx, the intensity of the bands was measured using the Image Lab 5.2.1 software (Bio-Rad, USA)

4.5.6 Protein chase assay

Cells (250 ml) were grown under standard conditions up to an optical density at 600 nm of 0.500. Then the culture was divided and left untreated or treated with either 0.5 mM diamide or 1 µg ml^{-1} vancomycin. Protein degradation was stopped by treatment with TCA (final concentration 10% TCA), and the amount of sample normalized by optical density. Cells were then washed twice with ice-cold acetone and led to dry for 10 min at room temperature. The pellet was resuspended into 100 µl of 1X Laemmli buffer. and 10 µl samples of the lysate were loaded in a 4-20% acrylamide gel. Protein degradation was then studied by western blot. For quantification of protein degradation, the western blot bands were measured using the Image Lab 5.2.1 software (Bio-Rad, USA), and normalized against the SDS-PAGE gel. For normalization, a total of 10 bands from the SDS-PAGE gel were quantified by densitometry and used to determine the amount of total protein loaded in each lane of the gel.

4.5.7 RNA isolation and northern blot

RNA isolation and northern blot were carried out as previously described (Rojas-Tapias and Helmann, 2018a; 2018b). RNA probes were synthesized from PCR products using *in vitro* transcription as previously described (Rojas-Tapias and Helmann, 2018a; 2018b). The template PCR product for the *clpC* RNA probe was generated using the primers DR309 and DR310, while for the *clpE* RNA probe we used DR302 and DR303.

4.5.8 Growth curves and spot dilution assays

For growth curves, cells (5 ml) were grown under standard conditions up to an optical density at 600 nm of ~0.500. Then, cells were resuspended at a final OD_{600}~0.01 in fresh sterile LB media supplemented or not with IPTG, and samples of 150 µL were placed in a 96-well plate. Optical density

at 600 nm was monitored for 20 h at 37ºC with continuous shaking in a Synergy H1 Microplate Reader (BioTek). For spot dilution assays, cells (5 ml) were grown under standard conditions up to an optical density at 600 nm of ~0.500, and serially diluted in sterile LB. The different dilutions were plated onto LB plates supplemented or not with IPTG.

4.6 Acknowledgments

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4.7 References

Antelmann, H., Scharf, C., Hecker, M., and Hecker, M. (2000). Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis. J Bacteriol *182*, 4478–4490.

Derré, I., Rapoport, G., and Msadek, T. (1999). CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in Gram-positive bacteria. Mol Microbiol *31*, 117–131.

Engman, J., and Wachenfeldt, von, C. (2015). Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. Mol Microbiol *95*, 51–63.

Fuhrmann, J., Schmidt, A., Spiess, S., Lehner, A., Turgay, K., Mechtler, K., Charpentier, E., and Clausen, T. (2009). McsB is a protein arginine kinase that phosphorylates and inhibits the heat-shock regulator CtsR. Science (New York, N.Y.) *324*, 1323–1327.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009). The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. J Bacteriol *191*, 1268–1277.

Haugen, S.P., Ross, W., and Gourse, R.L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. Nat. Rev. Microbiol. *6*, 507–519.

Kirstein, J., Dougan, D.A., Gerth, U., Hecker, M., and Turgay, K. (2007). The tyrosine kinase McsB is a regulated adaptor protein for ClpCP. Embo J *26*, 2061–2070.

Kirstein, J., MoliEre, N., Dougan, D.A., and Turgay, K. (2009). Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. Nat. Rev. Microbiol. *7*, 589–599.

Kommineni, S., Garg, S.K., Chan, C.M., and Zuber, P. (2011). YjbH-enhanced proteolysis of Spx by ClpXP in *Bacillus subtilis* is inhibited by the small protein YirB (YuzO). J Bacteriol *193*, 2133–2140.

Kruger, E., and Hecker, M. (1998). The first gene of the *Bacillus subtilis clpC* operon, *ctsR*, encodes a negative regulator of its own operon and other class III heat shock genes. J Bacteriol *180*, 6681–6688.

Larsson, J.T., Rogstam, A., and Wachenfeldt, von, C. (2007). YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. Mol Microbiol *66*, 669–684.

Leelakriangsak, M., and Zuber, P. (2007). Transcription from the P3 promoter of the *Bacillus subtilis spx* gene is induced in response to disulfide stress. J Bacteriol *189*, 1727–1735.

Leelakriangsak, M., Kobayashi, K., and Zuber, P. (2007). Dual negative control of *spx* transcription initiation from the P3 promoter by repressors PerR and YodB in *Bacillus subtilis*. J Bacteriol *189*, 1736–1744.

Lin, A.A., Walthers, D., and Zuber, P. (2013). Residue substitutions near the redox center of *Bacillus subtilis* Spx affect RNA polymerase interaction, redox control, and Spx-DNA contact at a conserved cis-acting element. J Bacteriol *195*, 3967–3978.

Mogk, A., and Bukau, B. (2003). MecA, an adaptor protein necessary for ClpC chaperone activity. Proc Natl Acad Sci USA *100*, 2306–2311.

Nakano, M.M., Hajarizadeh, F., Zhu, Y., and Zuber, P. (2001). Loss-of-function mutations in *yjbD* result in ClpX- and ClpP-independent competence development of *Bacillus subtilis*. Mol Microbiol *42*, 383–394.

Nakano, M.M., Nakano, S., and Zuber, P. (2002a). Spx (YjbD), a negative effector of competence in *Bacillus subtilis*, enhances ClpC-MecA-ComK interaction. Mol Microbiol *44*, 1341–1349.

Nakano, S., Erwin, K.N., Ralle, M., and Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. Mol Microbiol *55*, 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A.D., and Zuber, P. (2003). Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc Natl Acad Sci USA *100*, 13603–13608.

Nakano, S., Zheng, G., Nakano, M.M., and Zuber, P. (2002b). Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. J Bacteriol *184*, 3664–3670.

Newberry, K.J., Nakano, S., Zuber, P., and Brennan, R.G. (2005). Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. Proc Natl Acad Sci USA *102*, 15839–15844.

Nicolas, P., Nicolas, P., Mader, U., Mäder, U., Dervyn, E., Dervyn, E., Rochat, T., Rochat, T., Leduc, A., Leduc, A., et al. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science (New York, N.Y.) *335*, 1103–1106.

Oberto, J. (2013). SyntTax: a web server linking synteny to prokaryotic taxonomy. BMC Bioinformatics *14*, 4.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinova, A., Korelusova, J., Leduc, A., Bessieres, P., Dervyn, E., Krasny, L., and Noirot, P. (2012). Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. Nucleic Acids Res *40*, 9571–9583.

Rojas-Tapias, D.F., and Helmann, J.D. (2018a). Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*. Mol Microbiol *107*, 659–674.

Rojas-Tapias, D.F., and Helmann, J.D. (2018b). Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. PLoS Genet *14*, e1007531.

Tanner, A.W., Carabetta, V.J., and Dubnau, D. (2018). ClpC and MecA, components of a proteolytic machine, prevent Spo0A-P-dependent transcription without degradation. Mol Microbiol *108*, 178–186.

Trentini, D.B., Suskiewicz, M.J., Heuck, A., Kurzbauer, R., Deszcz, L., Mechtler, K., and Clausen, T. (2016). Arginine phosphorylation marks proteins for degradation by a Clp protease. Nature *539*, 48–53.

Turgay, K., Hahn, J., Burghoorn, J., and Dubnau, D. (1998). Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. Embo J *17*, 6730–6738.

Zhang, Y., and Zuber, P. (2007). Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. J Bacteriol *189*, 7669–7680.

4.8 Supplementary information

4.8.1 Supplementary figures

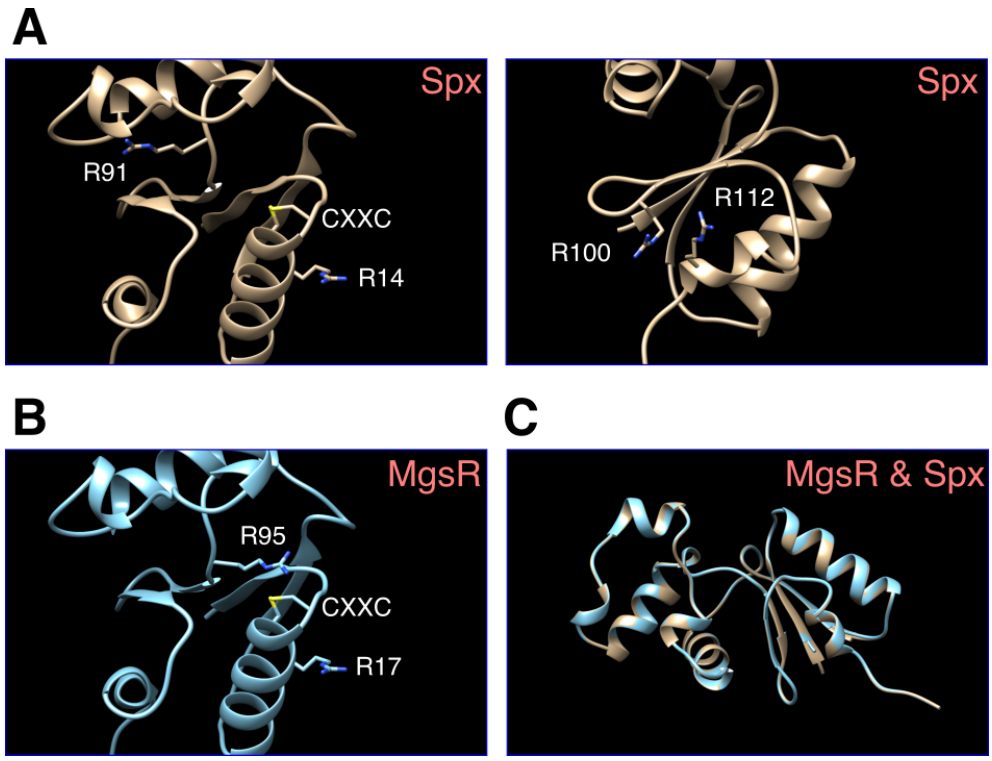

Fig S1. Putative phosphorylation of arginine residues on Spx and MgsR. A previous study showed phosphorylation of various arginines in Spx. A) Four residues were found to be phosphorylated in Spx. R91 has been shown to affect binding of Spx to RNA polymerase. R14 is located near the redox-sensing switch. R100 and R112 located are near the YjbH-binding site.

4.8.2 Supplementary tables

Table S1. Strains used in this study

Number	Genotype	Construction
HB18501	*WT*	Lab strain
HB23257	*spx::kan*	Lab strain
HB23259	*spx::tet*	Lab strain
HB18984	*ΔctsR*	Derivative of BKE00830 from BGSC. The erythromycin cassette was looped out using pDR244.
HB18986	*ΔmcsB*	Derivative of BKE00850 from BGSC. The erythromycin cassette was looped out using pDR244.
HB23043	*ΔywlE*	Derivative of BKE36930 from BGSC. The erythromycin cassette was looped out using pDR244.
HB18931	*clpC::ery*	gDNA of BKE00860 into WT.
HB23228	*ΔclpC*	Derivative of BKE00860 from BGSC. The erythromycin cassette was looped out using pDR244.
HB18930	*clpX::spec*	Lab strain
BKK28220	*clpX::kan*	BGSC strain.
HB18961	*clpP::tet*	Lab strain
BKK34540	*clpP::kan*	BGSC strain.
BKE13700	*clpE::ery*	BGSC strain.
HB18703	*yjbH::kan*	Lab strain
HB18658	*thrC::P_{trxB}-lacZ (ery)*	Lab strain
HB23017	*ΔctsR thrC::P_{trxB}-lacZ (ery)*	This work. gDNA of HB19658 was moved into HB19984.
HB23040	*ΔctsR amy::P_{hs}-ctsR (cm) thrC::P_{trxB}-lacZ (ery)*	A pPL82-based vector containing the *ctsR* coding region was transformed into HB23017, and therefore placed under IPTG control.
HB23041	*ΔctsR amy::P_{hs}-ctsR^{R63E} (cm) thrC::P_{trxB}-lacZ (ery)*	A pPL82-based vector containing the *ctsR^{R63E}* coding region was transformed into HB23017, and therefore placed under IPTG control. The point mutations were introduced by overlapping PCR with mutagenic primers.
HB23285	*spx::tet amyE::P_{hs}-spx (cm)*	A pPL82-based vector containing the *spx* coding region was transformed into HB23259.

HB23258	*ΔctsR spx::kan*	gDNA of HB23257 was moved into HB19984.
HB23260	*ΔctsR spx::tet*	gDNA of HB23259 was moved into HB19984.
HB23286	*ΔctsR spx::tet amyE::P_{hs}-spx (cm)*	gDNA of HB23285 was moved into HB23260
HB23287	*spx::tet amyE::P_{hs}-spx (cm) yjbH::kan*	gDNA of HB18703 was moved into HB23285
HB23288	*ΔctsR spx::tet amyE::P_{hs}-spx (cm) yjbH::kan*	gDNA of HB18703 was moved into HB23286.
HB23291	*spx::tet amyE::P_{hs}-spx (cm) clpP::kan*	gDNA of BKK34540 was moved into HB23285
HB23292	*ΔctsR spx::tet amyE::P_{hs}-spx (cm) clpP::kan*	gDNA of BKK34540 was moved into HB23286.
HB23268	*spx::kan amyE::P_{hs}-spx (cm)*	A pPL82-based vector containing the *spx* coding region was transformed into HB23257.
HB23272	*ΔctsR spx::kan amyE::P_{hs}-spx (cm)*	A pPL82-based vector containing the *spx* coding region was transformed into HB23258.
HB23363	*ΔmcsB spx::kan amyE::P_{hs}-spx (cm)*	gDNA of HB23257 was transformed into HB18986, and then the resulting strains was transformed with gDNA from HB23268.
HB23364	*ΔywlE spx::kan amyE::P_{hs}-spx (cm)*	gDNA of HB23257 was transformed into HB23043, and then the resulting strains was transformed with gDNA from HB23268.
HB23367	*ΔmcsB spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	gDNA of HB18930 was moved into HB23363.
HB23368	*ΔywlE spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	gDNA of HB18930 was moved into HB23364.
HB23281	*spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	gDNA of HB18930 was moved into HB23368.
HB23282	*ΔctsR spx::kan amyE::P_{hs}-spx (cm) clpX::spec*	gDNA of HB18930 was moved into HB23372.
HB23295	*spx::kan amyE::P_{hs}-spx (cm) clpX::spec clpC::ery*	gDNA of HB18931 was moved into HB23281.
HB23296	*spx::kan amyE::P_{hs}-spx (cm) clpX::spec clpE::ery*	gDNA of BKE13700 was moved into HB23281.
HB18807	*amyE::P_{hs}-spx^{DD} (spec)*	Lab strain
HB23278	*spx::kan amyE::P_{hs}-spx^{DD} (spec)*	DNA from HB18807 was moved into HB23257
HB23279	*ΔctsR spx::kan amyE::P_{hs}-spx^{DD} (spec)*	DNA from HB18807 was moved into HB23258
HB23280	*spx::tet clpX::spec*	gDNA of HB18930 was moved into HB23259.

BKE11520	*mecA::ery*	BGSC
HB18580	*mecA::ery spx::kan*	gDNA of BKE11520 was transformed into HB23257 and selected on LB supplemented with both erythromycin and kanamycin.
HB23019	*ΔmcsB thrC::P_{trxB}-lacZ (ery)*	gDNA of HB18658 was moved into HB18986.
HB23053	*ΔywlE thrC::P_{trxB}-lacZ (ery)*	gDNA of HB18658 was moved into HB23043.
HB23206	*ΔmcsB ΔywlE thrC::P_{trxB}-lacZ (ery)*	gDNA of HB18658 was moved into HB23199.
HB23199	*ΔmcsB ΔywlE*	gDNA from BKE36930 was transformed into HB18986, and the erythromycin cassette looped out using pDR244.
HB23346	*P_{spac}-mcsA-mcsB-clpC (spec)*	gDNA of HB18984 was used as template to amplify the upstream region of the *mcsA* gene, and then cloned into pMUTIN (spec). The plasmid was introduced into B. subtilis by single crossover. This vector places the *mcsA*, *mcsB*, and *clpC* genes under IPTG control at locus. This strain lacks ctsR.
HB23348	*spx::kan P_{spac}-mcsA-mcsB-clpC (spec)*	gDNA from HB23257 was moved into HB23346.
HB23333	*amyE::ctsR (cm)*	The coding sequence of *ctsR* as well as its promoter were cloned into pDG1662, and transformed into *B. subtilis*.
HB23352	*P_{spac}-mcsA-mcsB-clpC (spec) amyE::ctsR (cm)*	gDNA of HB23333 was moved into HB23346.
HB23353	*spx::tet P_{spac}-mcsA-mcsB-clpC (spec) amyE::ctsR (cm)*	gDNA of HB23259 was moved into HB23353.
HB23153	*ΔclpC amyE::P_{hs}-clpC (cm)*	The coding sequence of *clpC* was amplified by PCR and cloned into pPL82, and the plasmid transformed into *B. subtilis* WT. Then the *clpC::ery* cassette was transformed into the strain harboring the complementation cassette, and the erythromycin resistance gene looped out using pDR244.
HB23154	*ΔclpC spx::kan amyE::P_{hs}-clpC (cm)*	gDNA from HB23257 was transformed into HB23153.

** The ¨Δ¨* symbol is used to indicate a markerless deletion generated using the pDR244 plasmid on a BKE strain of the Bacillus Genomic Stock Center (BGSC).

Table S2. Primers used in this study

Number	Sequence
DR445	ATCACCCGGGGATGTTCATCCTACTAATTAGAGGAG
DR446	ATCGTCTAGACGAGCATTTATTACCAGCAGGTTG
DR315	ATCGACCCGGGGTCAAAGTCAGTAAAGGAGG
DR316	ATCGTCTAGAGACAAATCAATCTTTTCACCC
DR345	TTGTTGAGAGCAAAGAAGGGGGCGGCGGTTACATC
DR346	CCCTTCTTTGCTCTCAACAATATATCCTCTTT
DR450	ATCAGAATTCGTGAGAGTAGGACGCCGCCAAG
DR451	ATCAGGATCCGCACTCTTGACAAATCAATCTTTTCACCC
DR166	GGATAACAATTAAGCTTTCGGTAAAAGAAAAAGTGAGAC
DR366	GCTACAAGTTTACGAACCTGATCTTG
DR365	GTTCGTAAACTTGTAGCTGGACCTGGTG
DR342	ATCAGGTACCCAACATCATCAACTTTGCTGTTGG
DR343	ATCGAAGCTTTAGTCAAAGTCAGTAAAGGAGGG
DR364	ATCAGGTACCGAAAAGTGGCTGGTCTCTCG
DR309	CCACCGCGTTTCTATCACTG
DR310	ATCGATAATACGACTCACTATAGGGAGAGCCGCATCCTTCTCTTTACG
DR302	GTGGAAGCTGAGCACATTCA
DR303	ATCGATAATACGACTCACTATAGGGAGAGCTTCGTCTTACCGCTTTTG
6299	GCTTGTAAATTCTATCATAATTG
6300	AGGGAATCATTTGAAGGTTGG
6301	GCATTTAATACTAGCGACGCC

CHAPTER V: Perspectives

5.1 Summary

Previous research by the laboratories of Peter Zuber, Claes von Wachenfeldt, Kürsad Turgay, as well as our lab have elucidated the molecular pathways that result in activation of the Spx regulon in *B. subtilis* in response to disulfide, heat, and cell wall stress (Engman and Wachenfeldt, 2015; Garg et al., 2009; Jervis et al., 2007; Larsson et al., 2007; Leelakriangsak and Zuber, 2007; Leelakriangsak et al., 2007; Nakano et al., 2005; 2002b; Rojas-Tapias and Helmann, 2018a; 2018b; Runde et al., 2014; Zhang and Zuber, 2007). These works not only have uncovered the intricate regulatory network that governs the activation of the Spx regulon, but also its importance in response to stress conditions (Nakano et al., 2003; Rojas-Tapias and Helmann, 2018a; Runde et al., 2014). Spx is not only important in *B. subtilis*, but also in other Firmicutes, including several human pathogens, as it controls various stress responses and is required for survival under various conditions (Chapter I). The study of the function and regulation of Spx in *B. subtilis* and other organisms in therefore critical.

Although many of the regulatory mechanisms of Spx are now well understood in *B. subtilis*, several questions remain to be answered. This chapter aims at describing future areas of research in *B. subtilis* Spx regulation and function. These ideas emerged from my work at Prof. John Helmann's Lab.

5.2 Regulation of *spx* transcription

5.2.1 Role of σ^B in activation of the Spx regulon

The P_B promoter, located upstream the *yjbC-spx* operon, was shown to be induced in response to phosphate starvation (Antelmann *et al.*, 2000); however, the implication of this promoter in activation of the Spx regulon remains unknown. Since transcriptional activation alone seems not to be sufficient for sustained induction of Spx-controlled genes (Rojas-Tapias and Helmann, 2018b), it is

likely that upregulation of P_B is accompanied by Spx stabilization. In preliminary experiments, I observed that 0.35 M NaCl leads to upregulation of the *yjbC-spx* operon, which is consistent with transcriptomic data of *B. subtilis* growing in comparable conditions (Nicolas *et al.*, 2012). Furthermore, I observed that *trxB* was upregulated in response to salt stress, and that this activation also had a transcription-independent component, as cells with conditional *spx* expression were still able to upregulate *trxB*. Prior transcriptomic data also showed upregulation of Spx-controlled genes in response to salt stress (Nicolas *et al.*, 2012). Interestingly, phosphate starvation also results in *trxB* upregulation (Nicolas *et al.*, 2012). Further research will allow to understand the function of both P_B and Spx in the σ^B-mediated stress response.

5.2.2 Role of the YjbC putative acetyltransferase

Oxidative stress results in activation of *spx* at the P_A promoter, which is located in the intergenic region between *yjbC* and *spx*. This process is mediated by inactivation of the transcription factors PerR or YodB, which leads to derepression of P_A (Leelakriangsak *et al.*, 2007). By contrast, the transcriptional activation of *spx* in response to cell wall stress occurs at the P_{M1} promoter, which is located upstream the bicistronic *yjbC-spx* operon (Rojas-Tapias and Helmann, 2018a). This regulatory feature suggests that YjbC is important in the cell wall stress response, but not during oxidative stress conditions. YjbC is a putative acetyltransferase, however, its biological role is unknown. One possibility is that YjbC is important in the regulation of Spx through direct Spx acetylation; alternatively, YjbC might affect cellular pathways that are required during stress conditions. Indeed, cells lacking YjbC were shown to display increased sensitivity against ethanol, salt stress, and heat stress (Höper *et al.*, 2005). Its role during the cell wall stress if any remains to be investigated.

5.3 Regulation of Spx proteolysis

5.3.1 YirB-independent Spx stabilization in response to cell wall stress

When I was studying the stabilization of Spx under cell wall stress, I noted that even in the absence of the anti-adaptor YirB, Spx could still be stabilized in response to vancomycin (Rojas-Tapias and Helmann, 2018b). The mechanism associated to this stabilization is yet unknown. Some possible hypothesis that might explain this stabilization are presented. First, Spx may be stabilized by a decrease in ClpXP activity. This regulatory mechanism is important under disulfide stress and involves the oxidation of the cysteines in the ClpX Zn-binding motif. Whether cell wall stress leads to disulfide stress, and this stress to inactivation of ClpX has to be explored; 2) YjbH aggregation might also be a potential source of Spx stabilization. I performed some experiments and observed some YjbH aggregation after 30 min of vancomycin treatment, however, the biological relevance of this aggregation has to be studied; 3) stabilization of Spx by MecA and ClpC. Previously, it was reported that Spx can form a complex with MecA and ClpC *in vitro* (Nakano et al., 2002a). I hypothesize that this complex might be important to prevent ClpXP proteolysis.

5.3.2 Regulation of YirB by YuxN

YirB is critical to define Spx basal levels and induction of the Spx regulon in response to stress (Rojas-Tapias and Helmann, 2018b). *yirB* activation in response to cell wall stress conditions is mediated by the coordinated action of the CssRS two-component system and the TetR-like repressor YuxN. YuxN plays an important role in regulation of *yirB*, as cells lacking YuxN display a six-fold increase in *yirB* expression as well as elevated expression of Spx-controlled genes (Rojas-Tapias and Helmann, 2018b). The signals that result in YuxN activation are, however, unknown. Similarly, it is still unclear if YuxN also drives the expression of other genes in *B. subtilis*. Further research is thus

required to determine the biological conditions in which this repressor is important for regulation of *yirB* (and other genes), and consequently Spx.

5.3.3 Molecular mechanism of YuxN-mediated *yirB* repression

As previously stated, YuxN represses the expression of the *yirB* gene in *B. subtilis*. In addition, the expression of *yuxN* itself is also repressed by YuxN, which creates a negative feedback loop. The YuxN box at the *yirB* promoter is located at position -60 to -80 relative to the *yirB* start transcription site (i.e. YuxN Box I), and the YuxN box at the *yuxN* promoter overlaps its -35 box and is located at position +6 to +29 also relative to the *yirB* start transcription site (i.e. YuxN BoxII). Interestingly, I noted that deletion of any of the YuxN boxes led to derepression of *yirB*, suggesting that both boxes act cooperatively to repress *yirB* expression. Based on these results, we proposed a model in which YuxN leads to formation of a DNA repression loop, which precludes binding of the RNA polymerase to the *yirB* promoter. In this model, the loop is formed by formation of a dimer of YuxN dimers, with each dimer binding one YuxN box. Importantly, this model could explain how point mutations at the YuxN BoxI, which is located far upstream the RNA-binding site at P_{yirB}, can still affect *yirB* expression (Rojas-Tapias and Helmann, 2018b). Biochemical evidence for this model, however, is missing. The study of the DNA-binding properties of YuxN, the formation of multimeric YuxN structures, and the mechanism that results in the CssR~P-mediated derepression of YuxN *in vivo* must be investigated.

5.3.4 Regulation of Spx by MecA

In *B. subtilis*, as well as other Firmicutes, the *mecA* gene appears to be in close proximity to *spx*, suggesting an evolutionary relationship between both genes (Chapter I and IV). In the present work, we additionally elucidated the synthetic lethality of *mecA* and *clpX* in a *spx*-dependent fashion. However, the role of MecA in Spx regulation is still unknown. One hypothesis is that MecA is required

for Spx proteolysis through ClpCP, acting as an adaptor. Another hypothesis is that MecA stabilizes Spx and prevents its degradation through ClpXP (as mentioned above). Indeed, both hypotheses are not mutually exclusive since ClpCP-mediated Spx degradation occurs slowly compared to ClpXP-mediated proteolysis (Chapter IV), and they are supported by biochemical data that shows that 1) MecA can assist Spx degradation through ClpCP (Nakano et al., 2002b) and also that 2) MecA, ClpC, and Spx form a tripartite complex (Nakano et al., 2002a).

5.4 Post-translational modifications of Spx

5.4.1 Role of arginine phosphorylation in Spx regulation

In previous studies by Tim Clausen's lab and collaborators (Trentini *et al.*, 2016), it was shown that Spx was subjected to phosphorylation in four arginine residues. Those arginine residues are highly conserved in Spx homologs. Interestingly, the Spx paralog protein MgsR was also shown to be phosphorylated in almost identical residues, thereby suggesting a role for arginine phosphorylation in regulation of Spx proteins in *B. subtilis*. Further, during a transposon screening, we observed that cells lacking YwlE, the arginine phosphatase, displayed reduced induction of Spx-controlled genes (Chapter IV). This result was consistent with previous microarray experiments performed by Ulf Gerth and collaborators, where they showed that arginine phosphorylation impacts the induction of several regulons, including the Spx regulon (Elsholz *et al.*, 2012). Our evidence suggests that YwlE affects the induction of the Spx regulon, but the molecular mechanisms are yet not fully understood. One possibility is that YwlE directly modifies Spx at specific arginine residues. These modifications are likely to affect the capability of Spx to affect transcription by affecting the structure of the protein, its capability to bind DNA, or the acidity of the redox-sensing switch. Alternatively, direct modification of Spx might affect its degradation rate through both ClpXP and ClpCP. For instance, modification of the residue R112 might affect binding of Spx to YjbH, and therefore its degradation by ClpXP. The effect

of arginine phosphorylation on Spx might as well be indirect. For instance, the ability of ClpCP to degrade Spx might be enhanced under conditions that result in high levels of arginine phosphorylation; alternatively, the α-CTD domain of the RNA polymerase might be directly phosphorylated, which would affect the induction of the genes within the Spx regulon.

5.4.2 Role of the redox-sensing switch in activation of the Spx regulon

The Spx regulon is induced in response to disulfide and cell wall stress, however, both conditions result in differential post-translational modifications of Spx. While disulfide stress results in the oxidation of the redox-sensing switch located at the N terminus (Nakano et al., 2005), cell wall stress does not affect its oxidation state (Rojas-Tapias and Helmann, 2018a). Future research should thus address the question of whether the oxidation status of Spx affects the composition of the regulon. Additionally, we observed that the Spx mutant proteins Spx^{C10A} and $Spx^{C10AC13A}$ differentially affect the induction of the Spx regulon (Rojas-Tapias and Helmann, 2018a), which implies that oxidation of the switch is not essential for activation of Spx-controlled genes and that further mechanisms are at play.

5.5 Biological role of the Spx regulon

5.5.1 Activation of the Spx regulon in response to secretion stress

Full induction of the Spx regulon in response to cell wall stress requires the anti-adaptor protein YirB. Interestingly, the activation of *yirB* itself is mediated by the CssRS two-component system, whose importance was initially observed during secretion stress conditions (Hyyryläinen *et al.*, 2001). Since changes in the expression of *yirB* have significant effects on the induction of Spx-controlled genes (Kommineni *et al.*, 2011; Rojas-Tapias and Helmann, 2018b), it is highly likely that secretion stress also results in activation of the Spx regulon in a YirB-dependent fashion. A link between CssRS and Spx during secretion stress conditions has not yet been studied.

5.5.2 Function of the Spx regulon in the cell wall stress response

Cell wall stress leads to activation of the Spx regulon in a σ^M- and YirB-dependent fashion, and cells lacking Spx display increased sensitivity towards cell wall-active antibiotics (Rojas-Tapias and Helmann, 2018a; 2018b). The exact role of the Spx regulon within the cell wall stress response is yet unknown. The σ^M regulon is composed of ~60 genes and primarily induced in response to cell wall stress conditions (Helmann, 2016). Its regulon consist of several proteins involved in cell wall homeostasis. On the other hand, the CssRS regulon, which mediates activation of *yirB*, is composed by *yirB* itself and two more genes *htrA* and *htrB*, encoding two membrane-anchored serine proteases with important roles in protein quality control (Darmon *et al.*, 2002). The activation of Spx by these two regulons seems to support a model in which Spx might be important to prevent the formation of protein aggregates under conditions of elevated protein secretion, as observed during cell wall stress conditions. The role of Spx in the cell wall stress response is thus an important area of research.

5.5.3 Role of the thioredoxin system during cell wall stress

Cells lacking Spx are more sensitive to cell wall antibiotics. Interestingly, over the course of my experiments, I noted that overexpression of an artificial operon conformed by *trxA* and *trxB* in cells lacking Spx resulted in increased survival against cycloserine and ampicillin, but not fosfomycin (data not shown). This result, therefore, poses a model in which Spx is important during the cell wall stress response, since it allows the expression of both *trxA* and *trxB*. Additionally, it suggests that some proteins within the peptidoglycan biosynthesis pathway might be inactivated during oxidative stress conditions, and that Spx is important to alleviate this effect. Overexpression of the thioredoxin system, however, does not fully restore the phenotype of a Δ*spx* strain against cell wall antibiotics, and therefore other pathways are likely important.

5.5.4 Cells lacking YjbH display increased sensitivity towards cell wall antibiotics

In *B. subtilis*, Spx accumulation negatively affects many biological processes, including growth, competence, and sporulation (Larsson et al., 2007). Interestingly, I noted that cells lacking Δ*yjbH*, and therefore unable to degrade Spx, also displayed increased sensitivity to cell wall antibiotics. This implies that the control of Spx levels in the cell is critical, as cells lacking Spx also display increased sensitivity to cell wall antibiotics (Rojas-Tapias and Helmann, 2018a). One alternative is that Spx accumulation prevents the expression of genes involved in the synthesis of peptidoglycan by direct interaction with the RNA polymerase or by interfering with activator-stimulated transcription. Alternatively, Spx accumulation might affect the expression of genes involved in more general processes, such as translation or DNA repair, which might have broader implications. Further research will allow to uncover the mechanism associated.

5.6 References

Antelmann, H., Scharf, C., Hecker, M., and Hecker, M. (2000). Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis. J Bacteriol *182*, 4478–4490.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O.P., Devine, K.M., and Dijl, J.M.V. (2002). A Novel Class of Heat and Secretion Stress-Responsive Genes Is Controlled by the Autoregulated CssRS Two-Component System of *Bacillus subtilis*. J Bacteriol *184*, 5661–5671.

Elsholz, A.K.W., Turgay, K., Michalik, S., Hessling, B., Gronau, K., Oertel, D., Mader, U., Bernhardt, J., Becher, D., Hecker, M., et al. (2012). Global impact of protein arginine phosphorylation on the physiology of *Bacillus subtilis*. Proc Natl Acad Sci USA *109*, 7451–7456.

Engman, J., and Wachenfeldt, von, C. (2015). Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. Mol Microbiol *95*, 51–63.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y., and Zuber, P. (2009). The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. J Bacteriol *191*, 1268–1277.

Helmann, J.D. (2016). *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. Curr Opin Microbiol *30*, 122–132.

Höper, D., Völker, U., and Hecker, M. (2005). Comprehensive characterization of the contribution of individual SigB-dependent general stress genes to stress resistance of *Bacillus subtilis*. J Bacteriol *187*, 2810–2826.

Hyyryläinen, H.L., Bolhuis, A., Darmon, E., Muukkonen, L., Koski, P., Vitikainen, M., Sarvas, M., Prágai, Z., Bron, S., van Dijl, J.M., et al. (2001). A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. Mol Microbiol *41*, 1159–1172.

Jervis, A.J., Thackray, P.D., Houston, C.W., Horsburgh, M.J., and Moir, A. (2007). SigM-Responsive Genes of *Bacillus subtilis* and Their Promoters. J Bacteriol *189*, 4534–4538.

Kommineni, S., Garg, S.K., Chan, C.M., and Zuber, P. (2011). YjbH-enhanced proteolysis of Spx by ClpXP in *Bacillus subtilis* is inhibited by the small protein YirB (YuzO). J Bacteriol *193*, 2133–2140.

Larsson, J.T., Rogstam, A., and Wachenfeldt, von, C. (2007). YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. Mol Microbiol *66*, 669–684.

Leelakriangsak, M., and Zuber, P. (2007). Transcription from the P3 promoter of the *Bacillus subtilis spx* gene is induced in response to disulfide stress. J Bacteriol *189*, 1727–1735.

Leelakriangsak, M., Kobayashi, K., and Zuber, P. (2007). Dual negative control of spx transcription initiation from the P3 promoter by repressors PerR and YodB in *Bacillus subtilis*. J Bacteriol *189*, 1736–1744.

Nakano, M.M., Nakano, S., and Zuber, P. (2002a). Spx (YjbD), a negative effector of competence in *Bacillus subtilis*, enhances ClpC-MecA-ComK interaction. Mol Microbiol *44*, 1341–1349.

Nakano, S., Erwin, K.N., Ralle, M., and Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. Mol Microbiol *55*, 498–510.

Nakano, S., Küster-Schöck, E., Grossman, A.D., and Zuber, P. (2003). Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc Natl Acad Sci USA *100*, 13603–13608.

Nakano, S., Zheng, G., Nakano, M.M., and Zuber, P. (2002b). Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. J Bacteriol *184*, 3664–3670.

Nicolas, P., Nicolas, P., Mader, U., Mäder, U., Dervyn, E., Dervyn, E., Rochat, T., Rochat, T., Leduc, A., Leduc, A., et al. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science (New York, N.Y.) *335*, 1103–1106.

Rojas-Tapias, D.F., and Helmann, J.D. (2018a). Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*. Mol Microbiol *107*, 659–674.

Rojas-Tapias, D.F., and Helmann, J.D. (2018b). Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. PLoS Genet *14*, e1007531.

Runde, S., MoliEre, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., Gerth, U., Hecker, M., and Turgay, K. (2014). The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *Bacillus subtilis*. Mol Microbiol *91*, 1036–1052.

Trentini, D.B., Suskiewicz, M.J., Heuck, A., Kurzbauer, R., Deszcz, L., Mechtler, K., and Clausen, T. (2016). Arginine phosphorylation marks proteins for degradation by a Clp protease. Nature *539*, 48–53.

Zhang, Y., and Zuber, P. (2007). Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. J Bacteriol *189*, 7669–7680.